Development and Demonstration of Femtosecond Laser Micromachining Processes for Biomedical Applications

Dissertation

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Abstract

Microscale materials fabrication processes are necessary for many applications. Clean room techniques can be used to fabricate micro/nano scale device for biomedical applications, but they require masking and multiple steps as well as hazardous chemical reagents. One of the non-clean room techniques that achieves microscale resolution is laser ablation, specifically femtosecond laser ablation. Laser ablation by pulses with duration on the sub-picosecond or femtosecond time scales can remove materials with lower residual thermal effect, and the accuracy and quality of the device is often superior to conventional longer-pulse lasers. Also, it provides a convenient, economical and flexible way to fabricate programmable 3-dimensional patterns by varying the beam scanning speed during ablation as well as laser pulse energy. In addition, femtosecond laser ablation technique can be combined with other fabrication techniques as a primary or a post process.

In this dissertation, femtosecond laser micromachining technique was employed for microscale functional device fabrication with different materials to support biomedical researches. Femtosecond laser and material interaction properties, such as ablation threshold fluence, and incubation coefficient, were experimentally study for the bovine cortical bone and ethylene glycol dimethylacrylate (EGDMA) polymer. Ablation features were also characterized. Developed femtosecond laser micromachining was then used to fabricate microscale device for biomedical
applications. Micropillar on bovine cortical bone was made for micromechanical test purpose. Inlet/outlet microchannels were made on EGDMA polymer for cell loading. Array of microwells were patterned on blended electrospun poly(ε-caprolactone)/gelatin (PCL/gelatin) nanofiber scaffolds for tissue engineering application. Microchannels were made on electrospun poly(ε-caprolactone) (PCL) nanofiber scaffolds for vascular tissue engineering application. Sub-micron size of pores were drilled on polyvinylidene fluoride (PVDF) thin membrane for nanoelectroporation.
Dedication

Dedicated to my family
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Chapter 1

Introduction

1.1 Ultrashort pulse laser material processing

Ultrashort pulsed lasers that use regenerative amplification to produce optical pulses with durations less than 1 picosecond have recently been developed and made available for commercial materials processing applications [1,2]. A key characteristic of focused laser pulses produced by these systems is high peak laser intensity at low pulse energies. For example, a typical system of this type may produce optical-wavelength laser pulses with energy of 1 mJ and pulse width of 100 femtosecond ($10^{-13}$ s). When such a laser pulse is focused to a 1 µm spot diameter, the resulting peak intensity is on the order of $1 \times 10^{18}$ W/cm$^2$. Since most solid material are ionized by optical pulses with intensities in the range from $10^{13} \sim 10^{14}$ W/cm$^2$ [3], focused ultrashort laser pulses ionize and quickly remove absorbing materials from surfaces that they are focused on. Thus, ultrashort pulse lasers are effective tools for machining of surfaces with microscale resolution. Ultrafast pulsed laser machining with microscale to sub-microscale resolution is feasible in many different materials, including metals, polymers, dielectric materials, even biological tissues by pulsed laser ablation [1,4,5,6,7,8]. The process offers other benefits as well, including high precision, flexibility of pattern design, and non-contact
machining, that make the micromachining process feasible for various applications [9,10,11,12,13,14,15].

An important advantage of ultrashort pulsed laser ablation is that thermal effects on the machined surface are typically minimized. Because the femtosecond laser pulse duration is relatively short compared to the time required for energy to diffuse into the remaining surface, most of the absorbed energy is carried away in the ablated material. For example, in a metal with thermal diffusivity of $10^{-4}$ m$^2$/s, energy will only conduct into the surface to a characteristic depth of 3 nm during the 100 fs laser pulse duration.

Femtosecond laser micromachining is a convenient, cost-effective process since it does not typically require clean room conditions to achieve good pattern reliability. In chemical wet etching, small dust particles can block exposure of photoresist and produce corresponding pattern errors. In ultrashort pulse laser machining, such particles absorb negligibly small amounts of energy and are ablated with no effects on the accuracy of the machining process.

In research described in this dissertation, femtosecond (FS) laser micromachining processes were developed and employed to fabricate microscale devices to support biomedical device fabrication and other biomedical applications. Fabricated morphologies includes holes, micropillars, microwells and microfluidic channels. Some of these applications require machined features in materials that are difficult to structure by conventional fabrication techniques, due to material property limitations. For instance, bone is a hard, brittle material. Conventional mechanical machining is not suitable for micropillar fabrication on bone samples because the tool itself limits the achievable micropillar size. Also, friction heat and mechanical vibration during the mechanical
machining process can also limit the applicability of fabrication. Vacuum processes such as ion beam machining dessicate the bone sample, altering its properties. However, direct write femtosecond laser ablation can be used for precise machining bone in an atmosphere environment with minimal thermal effect on the bone surface.

Another application that is difficult to realize by other means is micromachining on polymer fiber materials. Micropatterning on electrospun scaffolds is important because it can be used for spatially-controlled cell distributions for tissue engineering applications. Since electrospun nanofiber scaffolds naturally have porous microstructures, it is hard to do micropatterning on the surface using conventional photolithography or mechanical machining processes. Femtosecond laser micromachining on porous nanofiber scaffolds is a unique process and can produce features on the surface with resolution only limited by the inherent porosity of the material.

Since femtosecond laser micromachining is a relatively new process, there is still a lack of knowledge about the laser ablation characteristics of many materials. Therefore, further studies to develop knowledge of laser and material interaction are needed to allow laser micromachining processes to be applied. The research described in this dissertation experimentally studied the femtosecond laser material ablation properties such as threshold fluence and incubation coefficient of selected materials including bovine cortical bone, blended electrospun poly(ε-caprolactone)/gelatin (PCL/gelatin) and PCL nanofibers, and ethylene glycol dimethacrylate (EGDMA) polymer. Ablated features and characteristics of machined surface were studied by optical and scanning electron microscopy. The chemical states before and after femtosecond laser ablation on PCL/gelatin scaffold were studied by X-ray photoelectron spectroscopy and attenuated
total reflection fourier transform infrared spectroscopy. An ultimate goal of the individual processes described in this dissertation was fabrication of microscale functional devices and features to support biomedical research. The function of the fabricated devices and structures were verified in collaborative work with other researchers at Ohio State University.

A titanium-sapphire RGA femtosecond laser (CPA-2161, Clark-MXR) system was used in all research described in this thesis. The full-width half-maximum pulse duration was 150fs and the wavelength was 775nm. This laser beam was delivered via series of laser optics and finally focused on the target materials using long-standoff microscope focusing objectives. With a coaxial vision system attached on the focusing optics in Z direction, the laser beam was focused on the target material by a micrometer and Z direction motion system. The laser beam was stationary and a computer numerical controlled linear X, Y, and Z motion systems with 500nm of resolution was used to move the machined substrates.

1.2 Organization of dissertation

In chapter 2, femtosecond laser material processing literature is reviewed. In chapter 3, fabrication of cylindrical micropillars on bovine cortical bone using direct-write femtosecond laser ablation is discussed. In chapter 4, experimental investigations aimed at assessing the effectiveness of femtosecond laser ablation for creating microscale features on electrospun poly(ε-caprolactone) (PCL)/gelatin nanofiber tissue scaffold capable of controlling cell distribution are described. In chapter 5, fabrication of arrays of microwells in ethylene glycol dimethacrylate (EGMDA) polymer is discussed. The wells are connected by nanoscale channels with sizes on the order of 10 nm using the DNA
Combing and Imprinting (DCI) technique. Fluorescent flow testing was performed to verify fluid connectivity between the laser-ablated filling channels and the microwell/nanochannel array. Chapter 6 discusses femtosecond laser ablation to produce microchannels inside electrospun polycaprolactone (PCL) scaffolds to direct bead seeding via fluid flows. Chapter 7 describes femtosecond laser application for Automated to Cell Biomolecule Analysis (ACBA) platform. Finally, chapter 8 summarizes results and makes some concluding remarks.
1.3 References


Chapter 2

Literature Review

2.1 Micro/nanoscale fabrication

It is necessary to have miniaturized structures for many applications. Not only are these applications the silicon technology in microelectronics [1], but also they are used for biomedical applications [2,3]. Micro or nanoscale widths and depths, and high aspect ratios of holes, grooves, etc. are required. The current existing fabrication techniques to create micro or nanoscale patterns are photolithography [4,5,6], soft lithography [7], stereolithography [8], and hot embossing imprint lithography [9]. Photolithography is a powerful way to create a high resolution pattern, but multiple steps with chemical solvents are required. Furthermore, residual chemical agents left on the surface could be harmful for bio applications. Soft lithography techniques can rapidly produce a microcontact printing at a low cost, but this technique is still restricted by development of appropriate masters. Stereolithography requires several steps to create three dimensional scaffolds. An advantage of hot embossing is that it can print a micropattern into organic materials at low cost, but it still needs a silicon master fabricated by standard micromachining techniques.
On the contrary, laser material process provides some advantages compared to other methods. This is one of non-clean room techniques and non-contact method. As a result, tool wear as in traditional mechanical machining is not a significant concern. Other benefits are single-step and flexibility of design. For this reason, laser material processes have been used for many different applications in industries for many years. Focused laser energy has been widely used for welding, cutting, drilling, engraving, and other modification of the materials. CO₂, Nd:YAG, copper vapor, and excimer laser systems have been conventionally used for laser machining [10,11,12,13]. Pulse-widths of these laser systems are usually ranged from continuous wave (CW) through milliseconds to hundreds of picoseconds. One problem for conventional laser material process is heat-affected zone (HAZ) in the base materials. This is because the pulse-width of most conventional laser systems are longer than the time required for thermal diffusion time of the most materials. Therefore, utilizing conventional laser system may lead to limitations for micro/nano scale laser material process.

2.2 Ultrashort pulsed laser material process

Ultrashort pulsed lasers overcome some shortcoming of long pulses laser system for micro and/or nanoscale fabrication. Pulse duration of these laser systems are typically less than 1 picosecond and such laser system are generically referred to as femtosecond lasers or ultrashort pulsed lasers. Typical laser-material interactions for the ultrashort pulse duration are quite different from the long-pulsed laser case. For ultrafast laser, ablation is occurred when the applied laser energy density surpasses a level related to the molecular binding and ionization energies of the target material [14]. That is, threshold of
energy density is required and it is typically called a laser fluence threshold \( (F_{th}) \) and the unit is energy per unit area (usually expressed as J/cm\(^2\)). This threshold value can be experimentally obtained. Details for finding this threshold fluence were described in chapter 3 later on. Figure 2.1 illustrates the laser ablation on the solid material. Laser fluence is distributed as a Gaussian shape. As can be seen, it is possible to create a laser ablated hole, which is smaller than the laser beam diameter, by adjusting \( F_0 \) very closed to \( F_{th} \).

![Laser fluence distribution with Gaussian profile](image)

Figure 2.1. Laser fluence distribution with Gaussian profile. \( F_0 \) and \( F_{th} \) are the peak laser fluence and the ablation threshold fluence, respectively. Beam diameter is \( 2w_0 \).

Shirk and Molian [15] reviewed the ultrashort laser interaction with solid materials. Electrons are excited by the absorption of photons when a laser beam is applied on a target material. By absorbing laser energy through collisions with ions, the electrons are heated to high temperature. The absorbed energy transferring to the lattice
by subsequent electron-phonon interactions requires order of picoseconds for most materials. Therefore, the time for energy transfer to the lattice is not sufficient for ultrashort pulse laser, resulting in minimum thermal damage on the substrate. Figure 2.2 depicts SEM images, showing comparison of long pulsed (nanosecond) laser ablation and femtosecond laser ablation with the 0.5mJ of laser pulse energy on metal substrate. Femtosecond laser ablation shows clean ablation at both edges with minimum or negligible heat affected zone (HAZ), while nanosecond laser ablation depicts much wider HAZ at side edges.

Figure 2.2. Comparison of nanosecond laser ablation (left) and femtosecond laser ablation (right) with the same pulse energy of 0.5mJ on metal substrate.
Before laser ablation is occurred, a significant amount of laser energy needs to be absorbed by the material. The absorption mechanisms can be different for absorbing materials, such as metals, semiconductors, and transparent dielectric materials (glasses and plastics) [16]. This absorption process can be a linear for metals or a nonlinear for transparent materials. For the linear absorption, it means that the electrons heated by photon absorption transfer the thermal energy to the lattice, resulting in melting or vaporization of the material [16]. On the other hand, the nonlinear process, such as multiphoton absorption, can become dominant especially for dielectric materials [16]. A bound electron can be lifted from its bound energy level or valence band to the free energy level or conduction band by simultaneously absorbing number of $m$ photons. That is, number of absorbed photons times photon energy should be equal or greater than ionization potential or bandgap [15,16]. For example, if the wavelength of laser is 775nm, the laser photon energy is calculated to be 1.56 eV. It requires at least 4 photons absorption for a material with 6 eV of ionization potential. For this reason, it is called multiphoton absorption.

There are two characteristic lengths for femtosecond laser ablation [16,17,18]. One is penetration skin depth, $l_s=1/\alpha$, where $\alpha$ is the absorption coefficient. For this case, the laser energy is only deposited in a surface layer based on absorption depth. Another characteristic length is the heat diffusion length, expressed as $l_d=\sqrt{D\tau_l}$, where $D$ is the heat diffusion coefficient and $\tau_l$ is the laser pulse width. This length is due to thermal conduction during the laser pulse. Mannion et al [18] characterized the optical penetration and heat diffusion length for various metals, such as stainless steel 316L, copper, niobium, and titanium, from the ablation rate (ablation depth per pulse) curves.
The optical penetration depths were ranged from 16 nm to 43 nm, while heat penetration depths were found from 52 nm to 103 nm for those metals. These characteristic lengths are dependent on laser parameters and material properties.

2.3 Applications using femtosecond laser ablation

Direct-writing femtosecond laser ablation has been enabled to fabricate complex three dimensional (3D) microfluidic devices for the extensive researches in the field of biotechnology applications with transparent materials [19,20,21,22]. This is due to the fact that focused ultrashort pulse laser beam has high enough peak intensity to drive nonlinear multiphoton absorption, consequently resulting in laser material processing in transparent substrates [23]. Various materials, such as silica glass [24], poly(methylmethaacrylate) (PMMA) [21,22] have been used for such devices.

Ultrashort pulse laser has been recently used for soft [14,25,26,27] and hard [28,29,30,31] tissue ablation in order to prevent dehydration as well as thermal damage to the surrounding tissue due to rapid temperature increase using continuous or a longer pulse laser due to the significant advantages over the longer pulsed laser. Advantages of the ultrashort laser for tissue ablation include efficient ablation due to small input of laser energy per ablated volume of tissue, minimal collateral mechanical damage due to short pulse duration of the stress impulse, minimal collateral thermal damage due to the extremely short deposition time, minimized pain due to localization of energy deposition, and ability to texture surface, and precise spatial control [32]. Schwab et al [29] investigated the temperature and pressure changes for middle ear surgery using femtosecond laser. For a given radiated power, the temperature and pressure changes for
femtosecond laser were significantly lower than Er:YAG and CO$_2$ lasers. Similarly, Neev et al [32] found that temperature increase did not exceed more than 2.5 °C for ablation of dentin. Girard et al [33] also studied effects of femtosecond laser irradiation on osseous tissues. Following laser ablation, they immediately stained alkaline phosphatase, which is a membrane-bound protein present on the surface of osteoblasts, to be used as a marker of heat deposition. It was shown that the alkaline phosphatase activity was seen in the cells bordering the edge of cut, indicating that there was no significant temperature increase.

There is another application of femtosecond laser ablation for soft tissue. Recently near infrared (NIR) wavelength of femtosecond pulsed laser systems have been used for the cell transfection to avoid cell damage at the UV and visible ranges of light. Stevenson, et al [34] demonstrated photoporation by using highly focused femtosecond laser beam (800nm of wavelength, 80MHz, 120fs, 0.85 NA) on the cells to transfetc macromolecules. It was reported that a focused spot size of 1μm was used for their experiment. They observed cells with perforated hole sizes between 0.8μm$^2$ and 3.9μm$^2$ were healthy, with no signs of cell blebbing. At the same time, they did analysis of transfection efficiency and cell viability using 4000 Chinese Hamster ovary (CHO) cells. They found that the maximum of 71% of transfection efficiency was achieved at the laser fluence ranges of 0.5–2.0 μJ/cm$^2$.

In conclusion, mechanism of femtosecond laser ablation was explained in the chapter 2. Also, two distinctive advantages, such as high peak intensity and minimal thermal damage, of ultrashort pulsed laser enable for microscale modification of many materials. For this reason, direct-write femtosecond laser micromachining was adapted
for the present work to fabricate microscale functional devices to support biomedical researches.
2.4 References


Chapter 3

Micropillar fabrication on bovine cortical bone by direct write femtosecond laser ablation

3.1 Introduction

Mechanical tools such as hand drills and cutting saws have been used to cut or drill hard tissues for laboratory analysis and for surgical operations, although the use of these tools has significant drawbacks such as high material loss, poor surface evenness, potential fragment contamination and significant tissue vibration [1]. Laser ablation is an attractive method to overcome the shortcomings of mechanical cutting tools. It is used in biomedical applications because it provides non-contact and precise removal of a variety of sensitive soft and hard tissue. In this work, we studied machining of bovine bone with a femtosecond pulsed laser.

Many different types of lasers with different wavelengths and pulsed output characteristics have been used to machine hard and soft tissues and the mechanisms and characteristics for tissue removal have been described in previous reports. Vogel and Venugopalan have presented an extensive review of the fundamental mechanisms of pulsed laser ablation of biological tissues [2]. Ultrashort pulsed lasers ablation of biological tissues and organs was modeled as a plasma-mediated process and the thermal,
mechanical, and chemical effects are summarized. As shown in this work and others as well [3], the depth of the thermally-damaged zone on the remaining surface generally increases with pulse duration. Ultrashort pulsed laser ablation using an has been recently used for soft [4,5,6,7] and hard [8,9,10,11] tissue ablation to minimize the thermal and mechanical damage of the surrounding tissues. Advantages of the ultrashort laser for tissue ablation include efficiency (small laser energy input per ablated volume of tissue), minimal depth of stress wave propagation due to the short duration of the pressure impulse and ability to texture a surface with precise spatial control [2]. Effects of femtosecond laser irradiation on osseous tissues were studied by Girard et al. [12]. Following laser ablation, they immediately stained alkaline phosphatase, a membrane-bound protein present on the surface of osteoblasts as a marker of heat deposition. Microscopy revealed alkaline phosphatase activity in the cells bordering the edge of cut, confirming damage during ablation was limited.

Since linear scanned ablation is required for cutting and texturing of surfaces, the results of investigators who have studied the effect of damage accumulation in non-biological materials due to repeated exposure to sub-ablation energy that is inherent in scanned ablation is relevant. Mannion et al [13] studied the effect of damage accumulation behavior using ultrashort laser pulse irradiation of common metals in air. Furthermore, Gomez et al [14] studied prediction of microchannel widths and depths ablated by focused femtosecond laser beam for polycarbonate (PC) and poly(methyl methacrylate) (PMMA). Choi et al [15] used incubation analysis similar to that described in this article for analysis of scanned ablation of thin films of conducting oxide. In all cases, ablation width and depth predictions based on analysis of change in material
ablation threshold due to damage accumulation showed good correlation to experimental measurements.

Our literature review shows that more research is needed to support the precise creation of three-dimensional microscale features on bone surfaces by femtosecond laser ablation. The structures of interest in this research are micropillars for microscale mechanical testing. The structure of bone is hierarchical in nature and the mineralized tissue that comprises bone has an anisotropy associated with it. The mechanical behavior of bone is governed by this structure and as a result is highly statistical [16]. Previous studies of other engineering materials, for example composites, have shown that microscale mechanical testing enables the prediction of macroscopic mechanical behavior [17]. Microscale mechanical testing of bone will be used in future research to delineate the microstructural components of bone in an effort to model the aggregate material. Bovine cortical bone micromechanical test specimens (micropillars) will be used in compression testing in an attempt to determine the mechanical behavior of the fundamental components of bone. Precise fabrication of such microscale features require prediction of the extent of ablation by analysis based on material ablation properties and effects of damage accumulation. One objective of this work is the experimental measurement of these properties for bovine cortical bone from single pulse ablation and linear scanned ablation experiments. Another objective is to formulate a method for calculating the diameter of ablated micropillars and to test its accuracy. Micropillars machined during this phase of the work will also demonstrate of the capability of FS laser ablation to create this type of structure in bovine cortical bone.
3.2 Materials and methods

3.2.1 Preparation of bovine cortical bone sample

Bone specimens were obtained from the cortical regions of bovine femoral bone. The specimens were sectioned using a diamond saw with water as a lubricant and were wet ground to produce flat parallel surfaces using a successive series of 600, 800, and 1200 grit SiC paper so that surface scattering from inhomogeneous tissue could be minimized. The flat and parallel specimens were attached to a glass slide with double-sided tape for femtosecond laser ablation.

3.2.2 Direct-write femtosecond laser ablation

Figure 3.1 shows the schematic of femtosecond laser systems for the experiment. Frequency doubled pulses from a mode-locked erbium-doped fiber laser intensified in a Ti:sapphire regenerative amplifier laser (CPA2161, Clark-MXR) were used. The maximum output power of the laser was $P_{av}=2.5$ W, the pulse duration was $T_p=150$ fs, the pulse repetition frequency was $f_P=3$ kHz, the wavelength $\lambda$ was 775nm, and the beam diameter was 5 mm. Laser beam power was adjusted by a series of optics, including thin-film polarizing beam splitters and $\frac{1}{2}$ waveplates. The attenuated laser beam was delivered by a beam mirror train through a mechanical shutter and then focused on the material. A 50x infinity corrected microscope objective lens with numerical aperture—$NA=0.42$ (M plan Apo NIR 50x, Mitutoyo)—was used for focusing the femtosecond laser beam. Attenuated laser power was measured by a power meter (PM100, Thorlab) placed under the laser focusing lens. The beam quality was $M^2=1.2$ in the horizontal Y direction and
$M^2=1.3$ in the horizontal X direction. A computer controlled motion system (MX80L, Parker) with a 0.5 μm resolution in the X, Y, and Z axes was used to position the sample and focus optic.

![Diagram](image)

**Figure 3.1** A schematic of femtosecond laser system, linear X,Y, Z axes motion stages, and focusing optics ($NA=0.42$)

In order to find ablation properties of the bovine cortical bone, single-pulse and linear scanned ablation tests were performed on the bone surface at varying pulse energies and scan speeds. Dimensions of the ablated features from these tests were used for calculations of the effective Gaussian beam radius, single-pulse ablation threshold fluence, and incubation coefficient of the bone material.
3.2.2.1. Single pulse ablation

The ablation threshold fluence of the bone material was calculated from the dimensions of single-pulse ablation spots made at varying pulse energies. The analysis is based on an assumption that a pulse with Gaussian radial fluence profile and with peak fluence greater than an ablation threshold value of $F_{\text{th}}$ will ablate material within a radius where the pulse fluence equals or exceeds the ablation threshold value. The radius at which the laser beam fluence equals the ablation threshold fluence can be found by solving the equation

$$F_{\text{th}} = F_0 \exp\left(-\frac{2r^2}{w_0^2}\right)$$  \hspace{1cm} (3.1)$$

where $w_0$ is the Gaussian beam radius and $F_0$ is the peak fluence, related to pulse energy $E_p$ by

$$F_0 = \frac{2E_p}{\pi w_0^2}$$  \hspace{1cm} (3.2)$$

Similarly

$$F_{\text{th}} = \frac{2E_{\text{th}}}{\pi w_0^2}$$  \hspace{1cm} (3.3)$$

where $E_{\text{th}}$ is the pulse energy corresponding to ablation threshold fluence. Rearranging Eq. (3.1) to solve for the diameter $D$ of the area near the center of the beam from which material is removed provides the relation

$$D^2 = 2w_0^2 \ln\left(\frac{F_0}{F_{\text{th}}}\right) = 2w_0^2\left[\ln(F_0) - \ln(F_{\text{th}})\right]$$  \hspace{1cm} (3.4)$$

Experimentally, single pulse ablation spots with measured diameter $D$ are produced by varying pulse energy over a range of values while focus spot radius is maintained at a fixed value. A semi-logarithmic plot of $D^2$ versus $F_0$ is created, and the ablation threshold
fluence is found by extrapolating the linear curve resulting from this plot to zero diameter
where $F_{th} = F_0$. An effective focus spot radius for the experiment can be calculated directly
from the ablation data since calculated focus spot sizes are subject to inaccuracy [18].
Using Eqs. (3.2) and (3.3) in Eq. (3.4) yields

$$D^2 = 2w_0^2 \ln\left(\frac{E_0}{E_{th}}\right)$$

(3.5)

Thus, the effective radius of the laser focus spot needed for fluence calculations in Eqs.
(3.2) and (3.3) can be calculated from the slope of a plot of experimental ablation
diameter-squared versus pulse energy data.

### 3.2.2.2 Scanned ablation around a circular path

When laser pulse energy is applied to the same surface location (number of pulses
$N$ is greater than 1), ablation occurs for pulse energy less than that corresponding to the
ablation threshold fluence for $N=1$ (single pulse). This effect, termed incubation [19], is
attributed to accumulation of damage or defects from individual pulses. The effect of
incubation of pulses of the same energy on ablation threshold is often quantified by a
power-law relationship of the form

$$F_{th}(N) = F_{th}(1)N^{\zeta-1}$$

(3.6)

where $N$ is number of pulses and $\zeta$ is the incubation coefficient.

It is necessary to consider the pulse overlap distance when applying incubation
analysis to scanned ablation experiments. Incubation analysis for linear scanning has
been reported in prior work [25]. In the present experiments, we consider the geometry
associated with scanning a laser focus spot of radius $\omega_0$ and pulse repetition frequency $f$
around a circular path of radius $R$ with scanning speed $s$, shown in Figure 3.2. For given
circular locus displaced from the scanning path by distance \( r \), the length of the circular arc spanning a focus spot for a given pulse is \( d(r) \) and the scan distance between successive laser pulses is \( s(r)/f \). Analogous to the linear scanning case, the definition of overlap \( O(r) \) for this geometry is

\[
O(r) = 1 - \frac{s(r)}{d(r)f}
\]  
(3.7)

and the number of pulses laser \( N(r) \) incident on a point lying on the locus is

\[
N(r) = \frac{d(r)}{s(r)f}
\]  
(3.8)

The angle \( \theta \) defined in Fig. 2 is calculated by laws of cosines

\[
\theta = \cos^{-1}\left( \frac{R^2 + (R - r)^2 - w_0^2}{2R(R - r)} \right)
\]  
(3.9)

The distance and speed needed to compute overlap and pulse number are

\[
d(r) = 2(R - r)\theta
\]  
(3.10)

and

\[
s(r) = (R - r) \frac{s}{R}
\]  
(3.11)

It is noted that the maximum overlap of successive pulses occurs on a circular locus with smaller diameter than the programmed diameter and is slightly larger than the straight line overlap value calculated with constant laser focus spot diameter \( d \) and scanning speed \( s \).

Ablated channel width \( D_N \) for scanned ablation with pulse number \( N \) can be calculated by combining Eqs. (3.4) and (3.6)
\[ D_N = \sqrt{2\omega_0^2 \ln\left( \frac{F}{F_{th}(1)N} \right)} \]  
(3.12)

Furthermore, Eq. (3.8) can be substituted into Eq. (3.12), resulting in

\[ D_N = \sqrt{2\omega_0^2 \ln\left( \frac{F}{F_{th}(1)(d(r)f/s(r))} \right)} \]  
(3.13)

If channel width is known from measurements, Eq. (3.13) can be used to solve for an unknown parameter such as incubation coefficient.
Figure 3.2 Geometry associated with scanning a laser focus spot with radius $\omega_0$ around a circular path of radius $R$ at a scan speed $s$. The overlap between the areas irradiated by successive laser pulses (suggested by the solid and dashed circles) is related to the incubation of damage caused by application of energy from numerous pulses to the same point. Based on the geometry, number of pulses incident on a point lying on a circular arc with spacing $r$ from the scan path was calculated and compared with the linear scanning case.

Figure 3.3 shows a schematic of the ablation pattern used for micropillar fabrication. A focused laser beam was scanned around concentric circular paths, with the innermost pass having a diameter equal to the programmed diameter of the pillar.

In this example, 100-$\mu$m-diam micropillars were fabricated by removing the surrounding material in three consecutive layers, each consisting of 45 concentric circular passes. The
laser beam focus location was shifted down by 10 μm for each subsequent layer.

Subsequently, various sizes of micropillars were made using different average laser powers and scanning speeds. The diameter and height of micropillars were measured from environmental scanning electron microscope (ESEM) images. The measured diameters at the top surface of the micropillars were compared with the diameters calculated using fluence from paraxial laser beam propagation.

Figure 3.3 Procedure for fabricating a micropillar by direct-write laser ablation. The inner-most pass of each layer had the same programmed diameter and the laser beam focus was shifted downward by 10μm to machine successive layers. The resulting diameter of the top of the pillar was less than the programmed diameter due to the ablation width of the first pass of the first layer and further reductions by the low-fluence pulses irradiating the upper surface of the pillar during machining of the lower layers.
3.2.3 Microscopy

An optical microscope (OM) (Leica MZ16, Leica) and an environmental scanning electron microscope (Philips XL-30 ESEM) were used to characterize the ablated bone surface. The specimen was removed from the glass slide after femtosecond laser ablation and attached to an SEM mount with silver conductive paint. The single pulsed, linear scanned ablation and micromachined pillar arrays were imaged with ESEM using a gas secondary electron detector (GSED) and wet mode. This mode replaces the residual gas in the chamber with H₂O (g), thereby allowing imaging of hydrated biological specimens. Image analysis software, Image J (NIH, USA) was used to measure the diameter of single pulsed ablated, width of linear scanned microchannel, and diameter of micropillar from the ESEM images.

3.3 Results

3.3.1 Single pulse ablation on the bovine cortical bone

To establish the proper values of pulse energy for ablation of the bovine cortical bone, single pulse ablation spots at varying pulse energies from 5 μJ to 0.33 μJ were produced by scanning the focused femtosecond laser beam over the surface of the bovine bone, as shown in Figure 3.4.
Figure 3.4 ESEM images of single pulse laser ablation at different pulse energies made on the bovine bone sample (a) $E_p=0.33 \, \mu\text{J}$, (b) $E_p=1.33 \, \mu\text{J}$, and (c) $E_p=4.66 \, \mu\text{J}$. 
Ablated diameters $D^2$ are plotted against pulse energy in Figure 3.5(a). Effective Gaussian beam radius $w_0$ was found to be 2.72 μm from the slope the curve. Next, laser fluence was calculated using the effective laser focus spot diameter and ablation diameter-squared versus laser fluence was plotted as shown in Figure 3.5(b). Single pulse ablation threshold fluence $F_{th}$ was calculated by extrapolating to zero ablation spot diameter based on Eq. (3.4). It has been found that logarithmic plots of femtosecond laser ablation squared diameter versus fluence for metal generally have two slopes which reflect a change in mechanism at high (“strong” ablation) and low (“gentle” ablation) pulse fluences [13]. The same behavior is observed in the cortical bone ablation data in Figure 3.5(b). The two slopes indicated in the plot extrapolate to strong ablation fluence ($F_{peak} > 2$ J/cm$^2$) of 2.6 J/cm$^2$ and gentle ablation fluence ($F_{peak} < 2$ J/cm$^2$) of 1.7 J/cm$^2$. 
Figure 3.5 (a) Ablated diameter$^2$ versus laser pulse energy for single-pulse ablation trials. Effective Gaussian radius ($w_0$) was found to be 2.72$\mu$m from the slope of the curve. (b) Single pulse ablation threshold was calculated by extrapolating the ablated diameter$^2$ versus laser fluence curve to zero diameter. Gentle ablation threshold fluence was calculated as $F_{th} = 1.69$ J/cm$^2$ and strong ablation threshold fluence was calculated as $F_{th} = 2.6$ J/cm$^2$.

3.3.2 Scanned ablation of bovine bone

Scanned ablation at different laser fluences and scanning speeds was conducted on the bovine bone and ablated microchannel widths were measured. Two different laser
fluences \((F=2.87\ J/cm^2\) and \(14.34\ J/cm^2\)) at scanning speeds varying from \(1.83\ mm/s\) to \(0.02\ mm/s\) were used to create linear microgrooves on the bone specimen. The widths of the microgrooves \(D_N\) were measured from ESEM images with examples being shown in Figure 3.6.

Figure 3.6 Linear grooves on the bovine bone sample were produced by scanned ablation at different speeds and two laser fluences (a) \(F=2.87\ J/cm^2\) (b) \(F=14.34\ J/cm^2\).
The widths of microgrooves depicted in Figure 3.6 are plotted with respect to scanning speeds in Figure 3.7. At $F=2.87 \text{ J/cm}^2$, the average widths ranged from 3.5$\mu$m to 2.24$\mu$m as scanning speed varied from 0.02mm/s (corresponding to $N=783.36$) to 1.83mm/s ($N=8.9$). The width at the scanning speed of 0.02mm/s was much larger than the diameter of a single pulse at the same fluence because of incubation. The solid lines in Figure 3.6 represent best-fit curves of Eq. (3.12) for laser fluences of $F=2.87$ and 14.34 J/cm$^2$. The previously determined values of $w_0=2.72$ and $F_{th}=2.6 \text{ J/cm}^2$ were used and an incubation coefficient value of $\xi=0.89\pm0.02$ minimized the error of the two curves to the corresponding data. Table 3.1 compares ablation threshold for bovine cortical bone as measured in this work with that of other hard tissues such as porcine cortical bone, dentin, and enamel. The incubated gentle ablation threshold fluences are comparable to the incubated ablation threshold fluences for the other materials and is particularly close to the value reported for porcine cortical bone [12].
Figure 3.7 Incubation coefficient, $\xi$, was found to be $0.89 \pm 0.02$ by minimizing the error between Eq. (3.12) and linear scanned ablation channel widths at varying scanning speeds and different laser fluences ($F=2.87$ and 14.34 J/cm$^2$).
<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>$F_{th}(1)$ (J/cm$^2$)</th>
<th>$F_{th}(N)$ (J/cm$^2$)</th>
<th>$\xi$</th>
<th>$T_p$ (fs)</th>
<th>$\lambda$ (nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine cortical bone</td>
<td>2.6 (strong)</td>
<td>1.56 (N=100)</td>
<td>1.22 (N=1000)</td>
<td>0.89±0.02</td>
<td>150</td>
<td>775</td>
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<td></td>
</tr>
<tr>
<td>Bovine cortical bone</td>
<td>1.69 (gentle)</td>
<td>1 (N=100)</td>
<td>0.79 (N=1000)</td>
<td>0.89±0.02</td>
<td>150</td>
<td>775</td>
</tr>
<tr>
<td>Porcine cortical bone</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Enamel</td>
<td>2.2±0.1</td>
<td></td>
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<tr>
<td>Enamel</td>
<td></td>
<td>0.7 (N=100)</td>
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<tr>
<td>Dentin</td>
<td></td>
<td>0.5 (N=100)</td>
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</tr>
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</table>

Table 3.1 Comparison of laser ablation threshold fluence measured in this work with that of other hard tissues from literature.

### 3.3.3 Micropillar fabrication by direct-write femtosecond laser ablation

ESEM images of micropillars, which had a programmed diameter of 100μm and were fabricated with 3 layers of pulses are shown in Figure 3.8(a) and (b). An optical microscope image of these micropillars is also shown in Figure 3.8(c). No charring of the bone specimen is observed, similar to previous work [12]. After completion of the 1st, 2nd and 3rd layers, the average diameter of the top surface of the pillar was measured as 94μm, 92.3μm and 91.9μm. The ablation width for each pass (defined as the difference between the programmed and measured diameters) was 6μm, 7.7μm and 8.1μm. Table 3.2 also summarizes the different diameters of micropillars fabricated at varying average laser powers and scan speeds.
Figure 3.8 Micropillars with programmed diameter of 100μm and height of 30μm fabricated on bovine cortical bone. Average laser power of 6 mW and scanning speed of 0.67 mm/s were used. (a) SEM image of a 3x3 micro pillar array (b) Magnified SEM image of micropillar. Micropillar diameter was measured as 91μm and height was 30μm. (c) Optical microscope image of micropillars. No charring the bone surface was observed.
Programmed pillar diameter ($\mu$m) | Laser power (mW) | Number of layers | Scan speed (mm/s) | Measurement of pillar ($\mu$m) | Ablation width ($\mu$m) | Prediction of ablation width ($\mu$m) |
---|---|---|---|---|---|---|
5 | 5.5 | 1 | 0.67 | 0 | 5 | >5 |
10 | 3.5 | 3 | 0.67 | 4.02±0.33 | 5.98±0.33 | 5.32±0.4 |
30 | 4.5 | 9 | 0.67 | 23.1±0.83 | 6.9±0.83 | 6.4±0.34 |
100 | 6 | 3 | 0.67 | 91.9±0.9 | 8.1±0.9 | 8.3±0.09 |

Table 3.2 Comparison of measured and predicted ablation widths for micropillars with programmed diameters ranging from 5$\mu$m to 100$\mu$m.

### 3.4 Discussion

#### 3.4.1 Comparison experimental and theoretical ablation thresholds

It is interesting to compare the experimental single pulse ablation threshold fluence to values predicted from theory. Ablation threshold for dielectrics was previously studied by Gamaly et al [21], who arrived at an expression for ablation threshold

$$F_{th}^{d} = \frac{3}{4} (\varepsilon_b + J_i) \frac{l_s}{A}$$  \hspace{1cm} (3.14)

where $\varepsilon_b$ is binding energy, $J_i$ is ionization potential, $l_s/A$ is a ratio of skin depth to absorption coefficient and $n_e$ is free electron density. Experimental ablation thresholds for dielectric materials such as silica [21] and glass [22] are reported to be reasonably approximated by this expression. Cortical bone [23] is composed of 70% inorganic material and 30% proteins. The inorganic phase of cortical bone consists mostly of hydroxyapatite (HA), $\text{Ca}_{10} \text{(PO}_4\text{)}_6 \text{(OH)}_2$. Based on other work, the average atomic number density [24] $n_e = 7.8 \times 10^{22}$ atoms/cm$^3$ was used for the free electron density. Since
binding energy and ionization potential for bovine cortical bone are not available from literature, it has been assumed that the average binding energy $\varepsilon_b$ is 5 eV and the average ionization potential $J_i$ for bone sample is 12 eV, typical values for dielectrics [24]. The ratio of $l_s/A$ was estimated as $2\omega/c=4\pi/\lambda (=6.1673\times10^{-6}$ cm at $\lambda=775$nm) because the absorption in the ionized dielectric occurs in a skin layer [21]. For $\lambda=775$nm and $T_p=150$ fs for current laser system, free electron density $n_e$ for HA was calculated based on the general solution of the time dependence of the number of free electron density defined by the rate equation [21,25]. The calculated free electron density $n_e$ is $1.96\times10^{23}$ cm$^{-3}$ at the intensity of $2.92\times10^{13}$ W/cm$^2$. This intensity is in the range where femtosecond pulses fully ionize a semiconductor target [21]. An ablation threshold fluence $F_{th}^d$ was calculated as 2.46 J/cm$^2$, which is similar value of single pulse laser threshold fluence of 2.6 J/cm$^2$ measured for the bovine cortical bone. Based on Eq. (3.14), unknown parameters for complex materials, such as the sum of the binding energy and ionization potential can be practically obtained from the experimentally measured ablation threshold.

3.4.2 Comparison of circular and straight-line scanned ablation

It is interesting to compare the pulse numbers predicted by Eq. (3.8) to straight-line values predicted using $d$ equal to measured laser focus spot diameter and $s$ equal to scan speed. Using the measured focus spot radius of 2.72$\mu$m and scanning speed of 0.67mm/s, the maximum pulse numbers calculated from Eq. (3.8) for various programmed radii are shown in Figure 3.9. For example, when machining around a programmed diameter of 10$\mu$m with a pulse radius of 2.72$\mu$m, the maximum pulse number is 25.87 vs. a straight line value of 24.46. However, it was found that there was
no significant increase of the incubated ablation when incubated ablation threshold was calculated. It is shown that threshold for circular scanning \( F(25.87)=1.82 \) vs. the linear scanning \( F(24.46)=1.83 \) using \( \xi=0.89 \). Also, it was shown that maximum number of pulse for circular scanning is converged into linear scanning case as radii of micropillars are increased. As a consequence, it is reasonable to use number of pulse for linear scanning case to calculate incubated ablation threshold to predict different diameters of pillars.

Figure 3.9 Pulse number for circular scanning compared to linear scanning with focus spot radius of 2.72\( \mu \)m and various pillar radius. For the smallest pillar diameter (10\( \mu \)m) used in this work, the pulse number predicted by circular scanning analysis was only slightly larger (1.25\%) than that predicted by linear scanning. It was found out that there was no significant decrease of incubated ablation threshold for the circular scanning compared to linear scanning.
3.4.3 Comparison experimental and calculated pillar diameters

Using the ablation relationships and material property measurements summarized above, it is possible to predict the pillar top diameters produced by given ablation process settings.

For a scanning speed of $s=0.67$ mm/s, the number of pulses applied to a single location along the scan is $N=24.5$. Using $\xi=0.89$ and $F_\theta=2.6$ J/cm$^2$, the corresponding incubated ablation threshold fluence is calculated to be $F_\theta(24.5)=1.83$ J/cm$^2$. The smaller ablation threshold implies that the channel width for scanned ablation is larger than the diameter of a single ablation spot.

When the laser beam focus was shifted down in a positive z-direction for machining of 2nd and 3rd layers, the defocused laser fluence applied to the top surface of the pillar resulted in further ablation. The fluence for a Gaussian beam which propagates along the z axis can be written as [26]

$$F(r,z) = 2 \frac{E}{\pi w^2(z)} e^{-\frac{2r^2}{w^2(z)}}$$

(3.15)

where $E$ is the laser pulse energy and $w(z)$ is the radius of the beam at the axial distance $z$ from the minimum focus location. The paraxial approximation, valid for $NA<<n$, is used to simplify the dependence of the fluence on the axial distance $z$. It is expressed as

$$w(z) = w_0 \sqrt{1 + \left( \frac{NA}{n} \frac{z}{w_0} \right)^2}$$

(3.16)

where $NA$ is numerical aperture of the lens, $n$ is index of refraction, and $w_0$ is the radius of a diffraction limited spot at the focus of a Gaussian beam. Experience has shown that predictions of ablation size are best done using the “effective” beam focus spot size
calculated from the single pulse ablation data according to Eq. (3.5). Thus, the laser beam radius $w_0=2.72\mu m$ obtained in this manner was used in calculations to predict the extent of ablation of the top surface of the bone sample. Figure 3.10(a) shows the Gaussian diameter of laser beam propagation in air near the minimum focus of $2.72\mu m$. At a distance of $20\mu m$ from the focus, the beam radius was almost $10\mu m$ and the fluence was about one tenth of the fluence at the focus. Figure 3.10(b) shows the plot of fluence in longitudinal and radial directions with $w_0=2.72\mu m$ and an average laser power of $6mW$ (parameters used to fabricate the micropillars).
Figure 3.10 Analysis of laser beam propagating in air in the z direction by paraxial approximation (a) laser beam radius (b) Laser beam fluence distribution in radial and longitudinal directions at average laser power of $P=6$ mW. Beam focus spot radius $w_0=2.72\mu m$ calculated from the slope of single pulse ablation experiments was used in this calculation.
Figure 3.11 Laser fluence distribution in radial direction at $z=0$ (top surface of pillar) with $w_0=2.72\mu m$, $P=6\,mW$. For the 1$^{st}$ pass ($z=0$), 2$^{nd}$ pass ($z=10\,\mu m$), and 3$^{rd}$ pass ($z=20\,\mu m$), the peak fluences are $F_{\text{peak}}=17.2, 5.1,$ and $1.63\,J/cm^2$. The incubated ablation threshold for each pass was calculated by Eq. (3.6) at scanning speed of $0.67\,mm/s$ (corresponding to $N=24.5$) and $\xi=0.89$. For the 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ passes, each incubated ablation threshold fluence is $F_{\text{th}}(24.5)=1.83\pm0.12\,J/cm^2$, $F_{\text{th}}(49)=1.69\pm0.14\,J/cm^2$, and $F_{\text{th}}(73.5)=1.05\pm0.09\,J/cm^2$.

To calculate ablation of the top of the pillar by successive layers of laser pulses, threshold fluence was decreased according to the power-law relationship shown in Eq. (3.6) with pulse number $N=24.5$ for each layer. Figure 3.11 shows laser fluence distribution in the radial direction at the top surface of bone sample ($z=0$) for the 1$^{st}$, 2$^{nd}$, and 3$^{rd}$ passes. The peak fluences for the 1$^{st}$ and 2$^{nd}$ layers were larger than $2\,J/cm^2$ so the strong ablation threshold was used to calculate incubated laser fluence. The incubated threshold fluence for the first layer was $F_{\text{th}}(24.5)=1.83\pm0.12\,J/cm^2$ and that of the second layer was $F_{\text{th}}(49)=1.69\pm0.14\,J/cm^2$. For the 3$^{rd}$ layer, the gentle ablation threshold was incubated with the same coefficient used for the strong ablation pulses resulting in a third-pass
threshold of $F_{th}(73.5) = 1.05 \pm 0.09 \text{ J/cm}^2$. It is noted that prior research has shown that incubation coefficients are the same for strong and gentle ablation thresholds for a metal material [27]. For analysis of ablation results, it is convenient to define ablation width as the difference in programmed and measured pillar diameter. The predicted ablation widths are compared with the experimental ablation width for each pass in Figure 3.12. Error between predicted and experimental ablation widths is within about 0.2µm for each pass.

Figure 3.12 Comparison of calculated and measured ablation width (programmed diameter minus the diameter of the top surface of the pillar) for successive layers. Predicted ablation width was well-matched with the measurements.

Figure 3.13 shows smaller micropillars with programmed diameter of 30µm and measured average diameter of 23.1µm. Machining of these tall pillars required 9 layers of
pulses at an average laser power of 4.5mW and scanning speed of 0.67 mm/s. The final diameter of the top of the pillar was measured to be 23.1µm, corresponding to an ablation width of 6.9µm. Using the outlined procedure above, the width was predicted to be 6.4µm. Still smaller micropillars with programmed diameters of 10µm and 5µm were also machined and predicted and measured ablation width results are summarized in Table 3.2. Error between predicted and experimental final ablation widths is within about 0.6µm.
Figure 3.13 Micropillars fabricated on bovine cortical bone by direct-write femtosecond laser ablation. Pillars had programmed diameter of 30μm, measured average diameter of 23.1μm after machining with 9 layers of pulses at an average laser power of 4.5 mW and scanning speed of 0.67 mm/s.

3.5 Conclusion

In summary, single pulse ablation and linear scanned ablation were conducted on a bovine cortical bone sample. Single pulse ablation threshold was measured from single pulse ablation features and incubation coefficient was measured from the width of grooves machined by scanned ablation. Calculations showed that the value of ablation threshold agreed with estimates from an analytical model ablation model based on molecular binding energies and heat capacity. Micropillars were fabricated by multiple layers of concentric circular femtosecond laser ablation passes. No oxidation of the bone
was observed for femtosecond laser machining, unlike results with nanosecond laser pulses. The width of the top surface of the pillar was measured after each layer of pulses was also calculated based on pulse fluence from a paraxial focus calculation, ablation threshold fluence and incubation factor for the bone sample. The calculated pillar diameters were well-matched to measurements, confirming that femtosecond laser ablation is a practical means for precision structuring of bone surfaces.

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3.7 References


Chapter 4

Micropatterning and characterization of electrospun Poly(ε-caprolactone)/gelatin nanofiber tissue scaffolds by femtosecond laser ablation for tissue engineering applications

4.1 Introduction

In tissue engineering, environmental factors such as topographical patterns and surface chemistry of tissue scaffold substrates critically affect cell adhesion, morphogenesis, differentiation, motility, and function [1,2,3,4,5,6]. Structuring of tissue scaffold surfaces with topological features having sizes on the order of 100 micrometers can provide directed tissue development [7,8,9,10,11,12]. Scaffolds of electrospun bioreorbable polymer such as poly(ε-caprolactone) (PCL) nanofibers have been shown to provide an extracellular matrix that facilitates cell proliferation and growth [13,14,15,16]. Moreover, it has been reported that nanofiber scaffolds of blended bioreorbable polymer and biomaterial (for example PCL/gelatin blends) have mechanical, physical and chemical properties comparable to pure PCL accompanied by improved degradation and biocompatibility [17]. It is critically important that any process used to create topological features preserve these desirable biological responses.

The commonly-used lithographic patterning techniques suitable for microscale structuring require multiple steps and deposition of residual chemical agents that render
them unsuitable for patterning on electrospun polymer/biomaterial blend nanofiber scaffold surfaces. Most recent work showed micropatterning of PLGA and P((LLA-CL) nanofiber scaffolds using UV photolithography. However, this process requires a photomask for patterning and several hours to degrade the nanofiber scaffold sufficiently to achieve micropatterning [18]. In contrast, laser machining is an attractive approach since it is non-contact and is normally completed in one step that does not require any additional chemicals or materials. A primary concern with applying laser machining processes to nanofiber tissue scaffolds is the potential for thermal effects on the bioresorbable polymers and biomaterials. Femtosecond (FS) lasers can minimize the thermal effects of laser machining in comparison with conventional lasers with nanosecond pulse durations because the femtosecond time scale for deposition of laser energy in the machined surface is short relative to thermal diffusion times in most materials. Consequently, optical energy is largely confined to the absorbing volume and material modifications resulting from thermal diffusion into the surrounding material is usually negligible [19,20]. When the energy density in the absorbing volume exceeds a level related to the molecular binding and ionization energies of the material [21]; the absorbing material is ablated from the surface, carrying much of the absorbed energy with it. Because of the precision and small thermal effect of this material removal mechanism, FS laser ablation is now commonly applied for soft and hard tissues [22, 23] and has been investigated for micromachining of various biomaterials including gelatin, collagen, and biodegradable polymers [24,25,26,27,28]. The direct-write ablation process is convenient and economical for fabrication of biomedical devices because it can
produce programmable shapes on surfaces with one processing step and sterility of the material can easily be maintained.

The availability of a process creating three dimensional (3D) microscale patterns on nanofiber scaffolds will enable tissue engineering applications that require precise placement of cells in an organized pattern on a substrate having geometric constraints [29,30,31,32,33].

The goal of the present work was to characterize the effectiveness of FS laser ablation for creating microscale structures on surfaces of blended PCL/gelatin ES nanofiber tissue scaffolds. Experimental measurements of the effects of laser ablation on nanofiber diameter and porosity and chemical state were compared for as-spun and laser-ablated nanofiber surfaces. Material removal rate and achievable pattern resolution of FS laser ablation were determined. Comparisons of growth rates of stem cells on as-spun and laser-machined ES nanofiber surfaces were also made as a direct measure of the effect of laser machining on biocompatibility of nanofiber surfaces and to the ability of laser-machined micropockets to control cell spreading and formation of cell aggregates of a specific size.

4.2 Materials and Methods

4.2.1 Electrospun PCL/Gelatin Nanofiber Scaffolds

A 6.7wt% solution of poly (ε-caprolactone) (PCL, Sigma-Aldrich, Inc.) with molecular weight \( M_w = 65,000 \), dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was prepared by stirring overnight at room temperature. Additionally, a 6.7wt% solution of porcine gelatin Type A (Sigma-Aldrich) in HFIP was prepared by stirring overnight at
room temperature. The two solutions were then combined together at a 1:1 weight ratio, stirred for two minutes and then placed in a 60cc syringe with a 20 gauge blunt tip needle and electrospun using a high voltage DC power supply (Glassman High Voltage, Inc.) set to -26 kV voltage, 20 cm tip-to-substrate distance and 15 ml/hr flow rate. A 7.6 x 7.6 cm sheet of electrospun polymer approximately 0.2 mm in thickness was deposited onto the glass coated with indium-tin oxide (ITO). A schematic of electrospinning of PCL/gelatin nanofiber scaffolds is shown at Figure 4.1. The electrospun sheet was then placed in a vacuum overnight to remove the residual HFIP.

![Schematic of electrospinning of PCL/gelatin nanofiber scaffolds.](image)

Figure 4.1 Schematic of electrospinning of PCL/gelatin nanofiber scaffolds.

The PCL/gelatin solution was also dripped onto 18 mm diameter glass coverslips spun at 1,000 RPM in a spin coater to achieve a thin film for ablation threshold
measurements of bulk material. The solvent evaporated immediately, leaving behind a smooth flat film of the polymer blend.

4.2.2 Direct-Write FS Laser Ablation

Frequency doubled pulses from a mode-locked erbium-doped fiber laser intensified in a Ti:Sapphire regenerative amplifier laser (CPA2161, Clark-MXR) were used. Laser wavelength was $\lambda = 775$ nm. The maximum average output power of the laser was $P_{av}=2.5W$, pulse duration was $T_p=150fs$, pulse repetition frequency was $f_P=3$ kHz and collimated beam diameter was 5mm. The beam quality factors were $M^2=1.2$ in the vertical Y direction and $M^2=1.3$ in the horizontal X direction. Laser beam power was adjusted by a series of thin-film polarizing beam splitters and $\frac{1}{2}$ waveplates. The attenuated laser beam was focused on the material with a 50x infinity corrected microscope objective lens having numerical aperture NA= 0.42 (M plan Apo NIR 50x, Mitutoyo). Attenuated average laser power was measured by a power meter (PM100, Thorlab) placed under the laser focusing lens. A schematic of femtosecond laser system is depicted in Figure 4.2

Sample motion and focus lens positioning was provided by a computer controlled motion system (MX80L, Parker) with 0.5 $\mu$m resolution in the X, Y, and Z axes. The system was programmed to scan the PCL/gelatin nanofiber scaffold under the focused laser beam to fabricate patterns on its surface. To establish the proper values of pulse energy for ablation of this polymer blend, single pulse ablation with varying laser pulse energies was performed on spin coated PCL/gelatin samples.
Next, linear grooves were produced by scanning the focused FS laser beam over the surface of electrospun PCL/gelatin nanofibers at various laser average power settings. All ablation of linear grooves was performed at a scanning speed of $s=20\text{mm/s}$. The average power of the FS laser was varied from 2mW to 30mW (the corresponding pulse energy range was from 0.67μJ to 10μJ). A critical parameter in scanned ablation experiments is pulse overlap $O_d$, which refers to the fractional length of intersection between locations of focus spots for consecutive pulses. It is related to pulse repetition frequency $f_p$, focus spot diameter $d_f$ and scan speed $s$ as

$$O_d = \left( 1 - \frac{s}{d_f f_p} \right)$$  \hspace{1cm} (4.1)
The pulse overlap for the initial linear scans was selected as $O_d = -1$ so that the ablation volumes produced by non-overlapping single pulses could be measured individually.

The lateral dimensions of features produced by this scanned ablation procedure were measured by a scanning electron microscope (SEM) (Sirion, FEI). The feature depths were measured from SEM images of cross sections prepared by FS laser cutting perpendicular to the scanning axis at several locations where the feature width and depth were at local maximums (coinciding with the center of a single laser pulse ablation volume). After suitable ablation energy and scan speeds were determined from this initial study, direct-write FS laser ablation was used to fabricate 4 by 4 square arrays of 200μm diameter microwells with center-center spacing of 400μm, as shown in Figure 4.3. An average laser power of 15mW (pulse energy of 5μJ) was selected based on the prior linear scan experiments. The X - Y motion system scanned the sample around circular paths at a scanning speed of 0.67mm/s. The programmed pattern used to machine 200μm diameter microwell consisted of 2 layers of 40 concentric circles each. The radial spacing of circles was 5μm in both cases. The machining was done in two layers to produce microwells with 80μm depth. After machining the first layer the focus position was adjusted downward by 40μm to ablate the second layer.
Figure 4.3 Schematic of 4 by 4 array of microwell with 200µm of diameter. Spacing between each microwell was 400µm.

4.2.3 ATR-FTIR and XPS Spectroscopy

Chemical analysis of PCL/gelatin nanofiber scaffolds (done before and after FS laser irradiation) was carried out by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy over the range of 4000–4000 cm⁻¹. FTIR spectra were recorded on a spectrometer (Nexus 470, Thermo Nicolet,) using a single reflection horizontal attenuated total reflection (HATR, Smart MIRacle, Thermo Nicolet) accessory.

X-ray photoelectron spectroscopy (XPS) was used to analyze the surface chemistry of PCL/gelatin nanofiber before and after FS laser irradiation. XPS (Axis Ultra HSA, Kratos Analytical) measurements were made using a monochromatic Al X-ray
source (13kV). A survey and narrow scan spectra were taken on both an as-spun surface and inside laser machined microwells.

### 4.2.4 Cell Culture

Mouse embryonic stem cells (mES cells) (CCE line, StemCell Technologies, Vancouver, Canada) were grown on gelatin-coated culture-wares and maintained in an undifferentiated state using Dulbecco’s Modified Eagle’s Medium (DMEM with 4.5 g/l D-glucose) supplemented with 15%(v/v) fetal bovine serum (FBS), 100U/ml penicillin, 100 mg/ml streptomycin, 0.1mM non-essential amino acids, 10 ng/ml murine recombinant leukemia inhibitory factor (LIF; StemCell Technologies, Vancouver, Canada), 0.1mM monothioglycerol, 2mM L-glutamine (Sigma Aldrich, St. Louis, MO) and 1mM sodium pyruvate (Invitrogen).

In order to compare the cell growth in the microwells and on the as-spun nanofiber surface, mES cells were seeded at the initial density of 5,000 cells/mm² in 200µm diameter microwells and on the as-spun nanofiber surfaces. Cell numbers were quantitatively measured by fluorescence imaging assay (AlamarBlue, Invitrogen) on days 1, 2 and 3. Three independent cell culture samples were measured for each day and this experiment was repeated three times, yielding a total of 27 growth rate measurements for each of the two types of surfaces. The maximum cell growth rate was calculated as the maximum slope during the exponential phase of the cell density versus time curve for each experiment [34].
4.2.5 Microscopy

SEM and confocal microscopy were used to observe the cell growth both inside the microwell and on the as-spun nanofiber surface. For SEM imaging, scaffolds with cultured stem cells were prepared by rinsing with phosphate buffered solution (PBS) three times for five minutes each. The cells were then fixed using a 4% paraformaldehyde solution for 1 hour at room temperature, followed by rinsing three times with 0.1M PBS/0.1M sucrose solution for 5 minutes each. The samples were then dehydrated with a graded series of ethanol solutions (50%, 70%, 80%, 95%, and 100%) for 10 minutes each. This was followed by a graded hexamethyldisilazane (HMDS) series consisting of 3:1 ethanol:HMDS, 1:1, and 1:3 at 15 minutes each and then pure HMDS and allowed to air dry.

To examine the nanofiber scaffold microstructures as well as spin coated films, samples were coated with 15 nm of osmium (OPC-80T, SPI Supplies) prior to viewing by SEM imaging. The use of osmium plasma deposition instead of gold or gold-palladium sputtering eliminated concerns regarding PCL melting and provided higher resolution images of the fiber surface. 10 kV of accelerating voltage was used for SEM.

For confocal microscopy (Zeiss-Meta 510, Carl Zeiss) imaging, cells were fixed in a 4% paraformaldehyde solution for 30 min at room temperature, and washed twice with 0.1M PBS. Cells were then incubated in 3μM propidium iodide (PI) nucleic acid staining solution for 30 minutes at room temperature prior to imaging.
4.2.6 Statistical Analysis

The differences between fiber diameter and pore size distributions on as-spun ES PCL/gelatin nanofiber surfaces and inside microwells fabricated by femtosecond laser ablation were evaluated. For statistical analysis, 100 random, unique measurements of fiber and pore diameters were collected from SEM images (Figs. 2C-D) of each of the two surface conditions using image analysis software (Image J, NIH, USA). Average and standard deviation of the fiber diameter and pore size distributions were calculated and the statistical differences between the fiber diameter and pore size distributions were evaluated using Student’s $t$-tests (JMP software, SAS, USA) based on these sample sets. The statistical difference between cell growth rates on as-spun ES PCL/gelatin nanofiber surfaces and inside microwells fabricated by femtosecond laser ablation were also evaluated by Student’s $t$-tests. A value of $P < 0.05$ was used to signify a statistically significant difference in both fiber diameter and pore size distribution and cell growth rate distributions.

4.3 Results

4.3.1 Single pulse ablation on PCL/gelatin thin film

To find ablation threshold fluence of PCL/gelatin samples, single pulse ablation was done on the spin coated PCL/gelatin samples. Figure 4.4 shows SEM image of single pulse ablation test performed on PCL/gelatin thin film. Laser pulse energies were ranged from 5µJ to 0.15µJ using 50x focusing lens. Ablated diameters $D^2$ versus laser pulse energy are plotted in Figure 4.5(a). Effective Gaussian beam radius $w_o$ was calculated to be 1.67µm from the slope of the curve. Then, laser fluence was calculated using the
effective laser focus spot diameter and ablation diameter-squared versus laser fluence was plotted in Figure 4.5(b). Single pulse ablation threshold fluence $F_{th}$ was calculated by extrapolating to zero ablation spot diameter, resulting in 1.16 J/cm$^2$.

Figure 4.4 Scanning electron microscopy (SEM) image of a series of single pulse ablation test on PCL/gelatin thin film. Laser pulse energies were varied from 5µJ to 0.15µJ using 50x focusing lens.
65

Figure 4.5 (A) Ablated diameter$^2$ versus laser pulse energy for single-pulse ablation trials. Effective Gaussian radius ($w_0$) was found to be 1.67µm from the slope of the curve. (B) Single pulse ablation threshold was calculated by extrapolating the ablated diameter$^2$ versus laser fluence curve to zero diameter. Ablation threshold fluence was calculated as $F_{th}=1.16$ J/cm$^2$.

4.3.2 Direct-write FS laser ablation on electrospun PCL/gelatin nanofiber scaffolds

SEM images of FS laser ablated grooves made by linear scanned ablation at various laser pulse energies are shown in Figure 4.6(a). As discussed below, the width and depth of the grooves were analyzed in more detail to determine suitable process parameters for machining of microwells. An example of an array of laser-machined microwells is depicted by the SEM image in Figure 4.6(B). SEM images of the surface of an as-spun PCL/gelatin nanofiber scaffold and the nanofiber scaffold surface at the bottom of a laser-machined microwell are shown in Figure 4.6(c) and (d). Fiber diameter distribution histograms and pore size distribution histograms measured from the SEM images are displayed in Figure 4.7(a) and (b). The statistics of the distributions are
summarized in Table 4.1. Using Student’s t-test, no significant differences were found between average fiber diameter ($P = 0.058$) and average pore size ($P = 0.134$) for an as-spun flat surface and a surface at the bottom of a microwell.

Figure 4.6 SEM images of electrospun PCL/gelatin nanofiber scaffolds showing: (A) Linear grooves produced by FS laser irradiation at varying pulse energies at a scanning speed of 20mm/s. Groove width decreased with laser pulse energy. (B) A 4 by 4 array of 200μm diameter microwells produced by direct write FS laser ablation. Average power of 15mW ($E_p=5\mu J$) and scanning speed of 0.67mm/s were used. (C) Magnified image of as-spun ES nanofiber. (D) Magnified image of ES nanofiber inside a 200μm diameter microwell. Some debris from ablated nanofibers was re-deposited at the bottom of the microwell during machining.
Figure 4.7 Fiber diameter and pore size distributions measured from images of an as-spun fiber surface and a laser-machined fiber mesh surface at the bottom of a microwell: (A) fiber diameter distribution. (B) pore size distribution. No significant differences were found between average fiber diameter ($P = 0.058$) and average pore size ($P = 0.134$) for an as-spun surface and a surface at the bottom of a microwell.
Table 4.1 Statistics of fiber diameter and pore size distributions for as-spun and FS laser ablated surfaces. No significant differences (at the level $P < 0.05$) were found between the two conditions for fiber diameter ($P=0.058$) and average pore size ($P=0.13$).

Average, maximum and minimum widths and depths of grooves in ablated electrospun nanofiber at different pulse energies are plotted in Figure 4.8(a) and (b). The average maximum groove widths increased from about 15μm to 50μm as pulse energy increased from 0.75μJ to 10μJ. Groove depths increased from 20μm to 65μm as pulse energy was increased from 3μJ to 17μJ. The specific energy $H$ of the ablation plotted in Figure 4.8(c) is the optical energy required to ablate material during machining of grooves by a scanned laser focus spot. It was calculated from the average laser power $P_{av}$, average groove width $w$, average groove depth $d$ and linear scan speed $s$ as

\[
H = \frac{P_{av}}{wds}
\]
Figure 4.8 Sizes and specific energy of features ablated in electrospun PCL/gelatin nanofiber using a 0.42 NA focusing objective and scan speed of 20 mm/s at various pulse energies (A): width, (B): depth, and (C): specific energy.

### 4.3.3 ATR-FTIR and XPS

Figure 4.9 displays ATR-FTIR spectra measured on PCL/gelatin nanofiber surfaces both before and after FS laser ablation. Infrared spectra for PCL-related stretching modes were observed on both surfaces [35]. These include 2949 cm⁻¹ (asymmetric CH₂ stretching), 2865 cm⁻¹ (symmetric CH₂ stretching), 1727 cm⁻¹ (carbonyl stretching), 1293 cm⁻¹ (C-O and C-C stretching), and 1240 cm⁻¹ (asymmetric COC stretching). Common bands of protein appeared at approximately 1650 cm⁻¹ (amide I) and
1540 cm\(^{-1}\) (amide II), corresponding to the stretching vibrations of C=O bond, and coupling of bending of N-H bond and stretching of C-N bonds, respectively [36]. The amide I band at 1650 cm\(^{-1}\) was attributable to both a random coil and \(\alpha\)-helix conformation of gelatin [37].

![ATR-FTIR spectra of PCL/gelatin nanofiber before and after FS laser ablation.](image)

The fiber at the bottom of the microwells fabricated by FS laser ablation showed no chemical group changes.

Figure 4.10 shows XPS survey scan spectra measured on PCL/gelatin nanofiber surfaces both before and after FS laser ablation. Identical carbon, oxygen and nitrogen signals were measured on both surfaces. Five major peak components with binding energy at 284.6eV for C-H species, at 286.2eV for C-O species, at 288.6eV for the O-C=O species, at 285.8eV for the C-N species and at 287.4eV for the O-C-N species [38].
The C 1s core-level detailed spectra measured from PCL/gelatin nanofiber surfaces before and after FS laser ablation are also identical.

Figure 4.10 Comparison of chemical groups before and after FS laser irradiation on PCL/gelatin nanofiber by XPS. Survey scan spectra for each C 1s, N 1s, and O 1s core-level as well as narrow scan spectra for the C-1s core-level are shown. C 1s narrow scan shows that there are no chemical state changes inside the microwell produced by FS laser ablation.

4.3.4 mES cell growth

SEM and confocal microscope images were used to compare the growth of the mES cells in microwells and on flat as-spun nanofiber surface as shown in Figure 4.11 and Figure 4.12. From the images, it is obvious that the spreading of cells cultured in the...
microwells was spatially confined to remain within the wells. This was an expected, desired effect of culturing the stem cells in microwells. AlamarBlue fluorescence imaging assay was used to quantify the growth rate of mES cells cultured inside the FS laser-machined microwells and on as-spun surfaces. As shown in Figure 4.13, there was no significant difference in the cell density between the two growth environments during the 1st ($P = 0.067$) and 2nd day ($P = 0.171$). Exponential growth rates ($\mu$) between days 1 and 2 (calculated as the difference between natural log of 1st and 2nd day densities divided by time) was $0.025 \pm 0.007 \text{ hr}^{-1}$ for cells in the microwell and $0.023 \pm 0.003 \text{ hr}^{-1}$ for cells cultured on the as-spun flat surface. There was no statistical difference ($P = 0.363$) for these day1-to-day2 growth rates, indicating FS laser machining did not have any significant side effects on the initial cell proliferation rate. However, a statistically significant difference ($P=0.019$) was observed for the cell density measurements on as-spun surfaces and in microwells for the 3rd day.
Figure 4.11 (A) SEM images showing mES cells cultured for 1 day in the 4 by 4 array of 200μm diameter microwells in PCL/gelatin nanofiber. (B) Magnified image of the area within the white box in figure (A) showing mES cells confined within a 200μm microwell.
Figure 4.12 Confocal microscope images showing distributions of mES cells cultured on the as-spun PCL/gelatin nanofiber surface (A, C, E) and in a 200μm diameter microwell (B, D, F) after 1, 2, and 3 days of growth. mES cells remained confined within the 200μm microwell but were non-uniformly distributed over a larger area on the as-spun flat surface.
Figure 4.13 Average and standard deviation of mES cell density from nine independent tests for each day of culture on as-spun nanofiber surfaces and in microwells. No statistically significant differences for cell density after the 1st ($P=0.068$) and 2nd ($P=0.172$) days were found. Also, cell growth rates on the two surfaces (log difference between one-day and two-day densities divided by time) were statistically identical being $0.025 \pm 0.007 \text{ hr}^{-1}$ for the microwell and $0.023 \pm 0.003 \text{ hr}^{-1}$ for the as-spun flat surface, ($P=0.36$). Statistically significant smaller cell density ($P=0.019$) in microwells after the 3rd day was attributed to confinement effects. (* means $P<0.05$). Data points are the average of $n=9$, error bars represent $\pm 1SD$.

### 4.4 Discussion

Ablation threshold fluence of the blended PCL/gelatin thin film was found to be 1.16 J/cm$^2$ and is comparable to the value of gelatin ablation reported by others [39].

From the images in Figure 4.6(c) and (d) and fiber diameter and pore size distribution histograms in Figure 4.7(a) and (b), nanofiber morphology at the bottom of the microwells was essentially the same as that of as-spun nanofiber scaffold surfaces.
Only very minor melting was observed on fibers at the bottom of the microwell, although some debris was re-deposited in the microwell. These results confirm that FS laser ablation can create useful microscale features on the blended polymer/biomaterial nanofiber surfaces without significantly altering the desirable fiber morphology.

For grooves produced by scanned ablation with a laser pulse energy of 10μJ, the average maximum ablated groove width was 50 μm. At pulse energies below 1.5J, the measurements of lateral dimensions of single-pulse ablation volumes were more variable because of the effects of the mesh porosity (average pore size of 6.35 μm and standard deviation of 5.69 μm) limited the measurement resolution. For this reason, the ablation width and depth curves in Figure 4.8(a) and (b) were not continued below 20 μm. Even this minimum measurable ablation groove width is much larger than the optical focus spot diameter of 3.34 μm. The measured groove depths were also similarly large in comparison to the calculated optical depth of focus of 3.46 μm. The laser focus spot diameter, pulse repetition rate and scanning speeds used for scanned ablation produced a focus spot overlap of -1, meaning that centers of the focus spot for consecutive laser pulses were separated by 1 Gaussian focus spot diameter (approximately 3.34μm) along the scan path. In spite of this large separation of successive optical pulse locations, the scanned ablation produced mostly continuous grooves.

The specific energy of the ablation is plotted in Figure 4.8(c). The average specific energy for scanned FS laser ablation was less than 1 J/mm³ for all pulse energies. This value is dramatically smaller than the value of 500 J/mm³ which has been found for scanned ablation of thin film oxide layers [40]. The larger specific energy for the thin film ablation resulted from the fact that ablated groove widths were on the order of the
focus spot volume dimension and were consequently much smaller than groove widths produced by scanned ablation of nanofiber scaffold surfaces. The pulse energy used for subsequent microwell machining ($E=5\mu J$) was selected as a compromise which yielded acceptable feature resolution (lateral resolution of approximately $30\mu m$ and a depth resolution of approximately $25\mu m$) and minimal fiber melting at some expense of material removal efficiency.

When analyzing removal of material from surfaces by focused FS laser pulses, it is customary to assume that ablation occurs when the incident radiant exposure on the material surface exceeds an ablation threshold value $[41,42,43]$. This ablation threshold can be related to a corresponding volumetric energy density threshold for material removal by one or more mechanisms and the absorption depth of the optical energy $[44,45]$. The present observation of ablated features that are many times larger than the focus spot dimension can be partly explained by scattering of laser energy within the porous nanofiber mesh, which is expected to be pronounced for such a fibrous material structure $[46]$. The results imply that the interaction volume of the scattered optical energy was considerably larger than the focus volume. However, another effect that also contributes to wider-than-expected ablation grooves is heating, melting and vaporization of fibers by energy radiating from plasma formed by laser-material interaction. Such plasma was clearly visible to the un-aided eye at pulse energies at the upper range of pulse energies used for machining in this work, and it was also observed that increased minor melting of fibers at the edges of the machined grooves was associated with the ablation experiments where plasma was visible. Heat input from such a plasma source is expected to promote fiber melting because the time duration of plasma formed during
femtosecond laser ablation is typically on the order 10’s to 100’s of nanoseconds, much longer than the femtosecond laser pulse [47,48].

ATR-FTIR spectra displayed in Figure 4.9, were essentially identical for surfaces analyzed before and after FS laser irradiation. This indicates that FS laser ablation induced negligible changes in the composition of the remaining PCL/gelatin fiber insofar as chemical bonds with IR resonances are concerned. The only notable difference in the two spectra is the CO$_2$ band at 2350cm$^{-1}$, which is present in the as-spun surface analysis but to a smaller extent in the ablated surface. This band corresponds to the asymmetric stretching (v3) mode of CO$_2$ and the difference in the two analyses is attributed to a discrepancy in background species calibration of the instrument.

Similarly, the close correspondence of the XPS spectra in Figure 4.10 shows that FS laser ablation produced no significant changes in the chemical states of carbon, oxygen and nitrogen on the scaffold surfaces. The fact that the nitrogen signals are identical is particularly interesting since these are contributed by the gelatin component of the nanofiber material.

Cell growth rates between 1 and 2 days were calculated for as-spun flat surfaces and laser machined microwells. The obtained cell growth rate is comparable with other work using microarrays for stem cell fate [49]. The SEM and confocal microscope images in Figure 4.11 and Figure 4.12 confirm that the microwells performed their intended function, confining the spreading of mES cells to remain within the microwells for up to 3 days of growth. The effects of the confinement on cell spreading were evident in the confocal microscope images in Figure 4.12 at days 1,2 and 3. The densities of cells cultured in the microwells were somewhat smaller than densities of cells cultured on the
flat surfaces for days 1 and 2, and the difference became statistically significant after 3 days of growth. This difference is interpreted as being due to the geometrical confinement effects of the microwells, which caused the cells cultured in the microwells to approach the stationary growth phase earlier than cells proliferating on the flat as-spun surface. Others have found similar growth rate trends for stem cells growing in a confined geometry as compared to cell cultures spreading on an unrestricted flat surface [50].

More importantly though, this data shows that cells can be confined to precise locations, sizes, and shapes through the use of laser ablated patterns on nanofiber scaffolds without any effects on cell viability. The ability to spatially control cell growth could be critical in the development of tissue engineering as multi-cell cultures and constructs become more prevalent. Living tissue is comprised of more than one type of cell and in order to recapitulate the \textit{in vivo} environment tissue engineers must be able to efficiently and reproducibly seed and proliferate cells in a biologically relevant design. This work has demonstrated that femtosecond laser ablation allows nearly any pattern to be produced to facilitate the spatial control of cells on a tissue scaffold in 3D.

### 4.5 Conclusions

In conclusion, results of this study indicate that direct-write FS laser ablation is a viable microscale fabrication technique for structuring of electrospun nanofiber surfaces of tissue scaffolds made from blends of PCL and gelatin. Measurements from SEM images showed that laser machined surfaces had the same fiber and pore diameters as original as-spun surfaces and the spatial resolution of the machining was only limited by
the porosity of the nanofiber structure. The specific energy of scanned ablation of nanofiber scaffold material in this study was significantly larger than that required for ablation of a non-scattering thin-film material found in previous research due to the fact that dimensions of grooves machined in nanofiber were much larger in comparison to the width and Rayleigh range of the laser focal volume. ATR-FTIR and XPS measurements showed that there were no detectable changes in the physical and chemical states for nanofiber surfaces inside FS laser machined microwells as compared to as-spun nanofiber surfaces. mES cells maintained the same initial cell growth rates inside the microwells and on as-spun nanofiber surfaces, demonstrating the FS laser did not affect the biocompatibility of the nanofiber surface. However, the spreading of the cultured cells was contained after 3 days of growth, demonstrating that the laser-machined microwells had the desired capability to control cell aggregate size. Finally, the measurements made in this work supports future studies by enabling the design of tissue engineering constructs and culturing protocols that control the growth and spreading of mES cells, resulting in cell aggregates of specified size.

### 4.6 Acknowledgements

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4.7 References


46. vande Hulst HC 1981 Light scattering by Small Particles (Dover, NY)


Chapter 5

Direct-Write Femtosecond Laser Ablation and DNA Combing and Imprinting for Fabrication of a Micro/Nanofluidic Device on Ethylene Glycol Dimethacrylate Polymer

5.1 Introduction

Recently, microfluidic devices have been extensively used for biomedical applications [1,2]. Microfabrication technologies, such as hot embossing [3], lithography [4] have enabled fabrication of such a microscale devices. However, these processes require multiple steps and chemical toxicity is a concern. Most recently, direct-write femtosecond laser ablation technique has demonstrated as a promising method for microfabrication of microfluidic devices in organic and inorganic materials [5,6,7]. Femtosecond laser ablation offers the advantages of being a programmable, non-contact, precise patterning process. Additionally, it can be used as a either a primary process or post process. When the energy density of focused femtosecond laser pulses exceeds a level related to the molecular binding and ionization energies of the absorbing substrate [8], the absorbing material is ablated from the surface, carrying much of the absorbed energy with it. Femtosecond (FS) laser micromachining minimizes the thermal effects in comparison with conventional nanosecond pulse laser machining because the
femtosecond time scale is short relative to thermal diffusion times in most materials. Consequently, optical energy is largely confined to the absorbing volume and material modification resulting from thermal diffusion into the surrounding material is usually negligible [9,10]. In the present work, direct-write femtosecond laser ablation was used as a post-process technique to produce inlet and outlet microchannels for microwell-nanochannel arrays produced with the DNA Combing and Imprint (DCI) method [11].

In the present work, linear scanned ablation with a focused femtosecond laser beam is required for microchannel fabrication on Ethylene glycol dimethacrylate (EGDMA) polymer surfaces. This prepolymer resin is low viscosity and can be solidified via UV induced cross-linking polymerization after processing. For this reason, EGDMA has been used with a DNA imprinting technique [11,12] for fabrication of nanochannel/microwell array. Arrays in this polymer are hydrophobic so organic solvents and molecules can be loaded for nanoscale fluid transport and chemical reactions. Alternatively, the array can also be easily converted to be hydrophilic, allowing convenient sample loading for biomedical applications. Various nanofluidic applications such as bioseparation and gene delivery can be carried out in confined nano-environment provided by this system. In addition, EGDMA has been extensively used as a cross-linker to copolymerize with other polymers for biomedical applications [13,14,15,16].

Researchers have studied the effect of damage accumulation (incubation) in organic and inorganic materials due to repeated exposure to sub-ablation energy pulses that is inherent in scanned ablation [17,18]. Gomez et al [19] studied effects of incubation on microchannel width and depth for scanned ablation in polycarbonate (PC) and poly(methyl methacrylate) (PMMA). Choi et al [20] used incubation analysis to
characterize the scanned ablation of thin films of conducting oxide. Lim et al [21] predicted the top diameter of micropillars machined in bovine cortical bone by direct-write femtosecond laser ablation. In all of these reports, it was found that ablation width and depth predictions based on incubated material ablation thresholds showed good correlation to experimental results.

For determination of optimum process parameters for precise fabrication of microscale features in the present application, it is necessary to predict ablation dimensions from analysis based on material ablation properties and effects of damage accumulation. However, the literature contains no reports of the femtosecond laser ablation properties of EGDMA polymer. Thus, one objective of this work was the experimental measurement of these properties for EGDMA polymer from single pulse ablation and single-pass linear scanned ablation experiments. Having found ablation properties for EGDMA, another objective was to create microchannels with multiple side-by-side overlapping linear scans and with channel width predicted by an analysis based on material ablation properties. A final goal was to verify the ability of fluid to pass from these laser-machined filling microchannels into a microwell/nanochannel array created by DCI.

5.2 Materials and methods

5.2.1 EGDMA sample preparation

Ethylene glycol dimethacrylate (EGDMA) (Sigma-Aldrich, St. Louis, MO) was used as a prepolymer resin for fabrication of nanofluidic devices. The material properties
of EGDMA are given in Table 5.1. Standard glass cover slips were used as a solid substrate for EGDMA fabrication. Cover slips were first treated with Piranha solution (70% H₂SO₄ : 30% H₂O₂) and then soaked in 2 wt% 3-trimethoxysilylpropyl methacrylate (Sigma-Aldrich, St. Louis, MO) in toluene for 12 hr to provide strong adhesion between the glass surface and EGDMA resin. The mixture of EGDMA (99 wt%) and Irgacure 651 (Sigma-Aldrich, St. Louis, MO) photoinitiator (1 wt%) was loaded on treated cover slip glasses and cured with UV light for 20 minutes under nitrogen. Flat molds made of polydimethyl siloxane (PDMS) were used to produce flat EGDMA surface by imprinting.

<table>
<thead>
<tr>
<th>Molar mass (g mol⁻¹)</th>
<th>Density (g cm⁻³)</th>
<th>Boiling temperature (°C)</th>
<th>Viscosity at 20 °C (mPa·s)</th>
<th>Specific heat (cal g⁻¹ °C⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>198.22</td>
<td>1.051</td>
<td>98-100</td>
<td>3.2</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Table 5.1 Material properties of EGDMA.

Nanofluidic EGDMA devices were fabricated by the DNA combing and imprinting (DCI) technique [11], as shown in Figure 5.1. Briefly, regular arrays of PDMS pillars having diameter of 7 μm and center-to-center spacing of 8.2 μm were formed by imprinting. Aligned DNA nanowires were suspended across the tops of the PDMS with micropillars by a de-wetting process. Then, the nanowires and micropillars were sputter-coated with gold to form removable nanostructure. By an EGMDA resin imprinting and gold etching procedure, arrays of micro-wells and nano-channels were formed.
5.2.2 Direct-write femtosecond laser ablation experiment setup

A schematic of experimental setup is shown in Figure 5.2. Frequency doubled pulses from a mode-locked erbium-doped fiber laser intensified in a Ti:sapphire regenerative amplifier laser (CPA2161, Clark-MXR) were used for ablation. The maximum output power of the laser was $P_{av}=2.5$ W, the pulse duration was $T_p=150$ fs, the pulse repetition frequency was $f_P=3$ kHz, the wavelength was $\lambda=775$nm, and the beam diameter was 5 mm. Laser beam power was adjusted by thin-film polarizing beam splitters and $\frac{1}{2}$ waveplates. After attenuation, the beam polarization was rotated with a $\frac{1}{2}$ waveplate so that the polarization axis normal to the scan direction. The attenuated laser beam was delivered through a mechanical shutter and to the focusing optic by a series of reflected mirrors. The final delivery mirror had a dichroic front surface coating which
allowed a camera image of the work surface to be formed from light passing back through
the focusing optic. A 50x infinity corrected microscope objective lens with numerical
aperture $NA = 0.42$ (M plan Apo NIR 50x, Mitutoyo) was used for focusing the
femtosecond laser beam. Attenuated laser power was measured by a power meter
(PM100, Thorlab) placed under the laser focusing lens. A computer controlled motion
system (MX80L, Parker) with a 0.5 μm resolution in the X, Y, and Z axes was used to
position the sample and focus optic.

Figure 5.2 Schematic of direct-write femtosecond laser ablation experimental setup.

In order to determine the femtosecond laser ablation properties of the EGDMA,
single pulse and linear scanned ablation tests were performed on the polymer surface at
varying pulse energies and scan speeds. Dimensions of the ablated features from these tests were used in calculations to estimate the effective Gaussian beam radius, single pulse ablation threshold fluence and incubation coefficient of the EGDMA material. Next, linear microchannels were produced on pre-patterned microwell/nanochannel arrays by femtosecond laser ablation with 10 side-by-side multiple passes for each microchannel. The linear scan speed was 0.33 mm s\(^{-1}\), the laser power was 3 mW and the spacing between each linear scan pass was 1 \(\mu\)m. The length of the overlapping linear scan passes was adjusted to produce a 20 \(\mu\)m converging microchannel.

### 5.2.3 Rhodamine dye flow test

In order to verify that nanochannels remained open after fabricating the microchannel by direct-write femtosecond laser ablation, a fluorescence dye flow test was employed. Figure 5.3 contains a schematic of this Rhodamine flow test. A PDMS thin film (consisting of a 25:1 ratio of PDMS prepolymer and curing agent) was treated by oxygen plasma for adhesion and then used as a lid to seal the converging microchannel with no detectable leakage. Then, the micro/nanofluidic device was prefilled with mixture of ethyl alcohol and water (1:1) to remove the air bubbles trapped inside micro/nanochannel array. Finally, the 20 \(\mu\)m converging microchannel made by femtosecond laser ablation was filled with Rhodamine dye (10\(^5\) mol L\(^{-1}\)) water by pipette and 5-12 hours was allowed for the Rhodamine to diffuse into the device before imaging.
Figure 5.3 A schematic of Rhodamine dye flow test. A converging microchannel was machined on the EGDMA that had been pre-patterned with a microwell/nanochannel array by DCI. This sample was filled with Rhodamine dye solution, which diffused into the microwells/nanochannel array. The array was imaged by confocal microscopy to verify the fluid connectivity of nanochannels and the converging microchannel.

5.2.4 Microscopy

To examine ablated EGDMA polymer surface, samples were coated with 15 nm of osmium (OPC-80T, SPI Supplies) prior to viewing by SEM imaging. 10 kV of accelerating voltage was used for SEM (Sirion FEI). Image analysis software, Image J
(NIH, USA) was used to measure the diameter of single pulsed ablated, and width of linear scanned microchannel, from the SEM images.

High-speed confocal microscopy was used to obtain flow test on the fabricate pattern. The high-speed confocal microscope system consists of an Olympus IX-81 inverted microscope equipped with the Yokogawa CSU-22 confocal spinning disk system. The CSU-22 allows up to 1000 frames per second temporal resolution. 100x (NA=1.4) oil immersion objective lens was used for fluorescent images of nanochannels and microchannels.

5.3 Results and discussion

5.3.1 Pre-patterned EGDMA by DCI technique

Figure 5.4 shows a SEM image of patterned array of microwells and nanochannels on EGDMA polymer by DCI technique. The measured width of microwell was 7μm. A space between each microwell was measured to be 8.2 μm. By using DCI, it is shown that a large array of microwell and nanochannel that is laterally ordered form cab be produced. However, it is also necessary to create additional microgrooves for inlet and outlet channels which are interconnected microwell arrays for external loading foreign molecules or desired cells for furthermore biological experiment. Therefore, next step is to create microchannel using direct write femtosecond laser ablation on the prepatterned EGDMA.
5.3.2 Single pulse ablation threshold and incubation coefficient

Single pulse ablation spots were made on an EGDMA surface and were used to measure the ablation threshold fluence $F_{th}$ of the polymer. The analysis assumes a focus spot with Gaussian radial fluence profile and a maximum fluence greater than the ablation threshold. The radius at which the laser beam fluence equals the ablation threshold fluence can be found by solving the equation

$$ F_{th} = F_0 \exp \left( -\frac{2r^2}{w^2_0} \right) $$

where $w_0$ is the Gaussian beam radius and $F_0$ is the peak fluence, related to pulse energy $E_p$ by the expression

$$ F_0 = \frac{2E_p}{\pi w_0^2} $$
Similarly

\[ F_{th} = \frac{2E_{th}}{\pi w_0^2} \]  

(5.3)

where \( E_{th} \) is the pulse energy corresponding to ablation threshold fluence. Rearranging Eq. (5.1) to solve for the diameter \( D \) of the spot near the center of the beam from which material is removed provides the relation

\[ D^2 = 2w_0^2 \ln\left(\frac{F_0}{F_{th}}\right) = 2w_0^2 [\ln(F_0) - \ln(F_{th})]. \]  

(5.4)

Experimentally, single pulse ablation spots with measured diameter \( D \) are produced by varying pulse energy over a range of values while focus spot radius is maintained at a fixed value. A semi logarithmic plot of \( D^2 \) versus \( F_0 \) is created, and the ablation threshold fluence is found by extrapolating the linear curve resulting from this plot to zero diameter where \( F_{th} = F_0 \). An effective focus spot radius for the experiment can be calculated directly from the ablation data since calculated focus spot sizes are subject to inaccuracy [22]. Using Eqs. (5.2) and (5.3) in Eq. (5.4) yields

\[ D^2 = 2w_0^2 \ln\left(\frac{E_0}{E_{th}}\right). \]  

(5.5)

Thus, the effective radius of the laser focus spot needed for fluence calculations can be calculated from the slope of a plot of experimental ablation diameter-squared versus pulse energy data.

To establish the proper pulse energy values for ablation of EGDMA, single pulse ablation spots at were produced at pulse energies varying from 5 µJ to 0.5 µJ by scanning the focused femtosecond laser beam over the surface of the EGDMA polymer. The diameters of the ablation spots were measured from the SEM images Figure 5.5.
Figure 5.5 SEM image of single-pulse laser ablation at different pulse energies made on the EGDMA polymer.
Figure 5.6 (a) Squared ablated diameter versus laser pulse energy for single-pulse ablation trials. Effective Gaussian radius ($w_0$) was found to be 2.95 µm from the slope of the curve. (b) Single-pulse ablation threshold was calculated by extrapolating the ablated diameter squared versus laser fluence curve to zero diameter. Ablation threshold fluence was calculated as $F_{th}=3.76 \text{ J cm}^{-2}$.

Ablated diameters $D^2$ are plotted against pulse energy in Figure 5.6(a). Effective Gaussian beam radius $w_0$ was found to be 2.95 µm from the slope the curve. Laser fluence was next calculated using the effective laser focus spot diameter and ablation diameter-squared versus laser fluence was plotted as shown in Figure 5.6(b). Single pulse ablation threshold fluence $F_{th}$ was calculated by extrapolating to zero ablation spot diameter based on Eq. (5.4), resulting in $F_{th} = 3.76 \text{ J cm}^{-2}$. This ablation threshold fluence
is somewhat larger than some other polymers, such as 2.6 $J \ cm^{-2}$ for PMMA [9], but it is lower than the ablation threshold of 4.6 $J \ cm^{-2}$ for PDMS [23].

When the same laser pulse energy is applied to the same surface location (number of pulses $N$ is greater than 1), ablation occurs for pulse energy less than that corresponding to the ablation threshold fluence for a single pulse ($N=1$). This effect, termed incubation, is attributed to accumulation of damage induced by individual pulses [17]. The effect of incubation on ablation threshold is often quantified by a relationship of the form

$$F_{th}(N) = F_{th}(1)N^{\xi-1}. \quad (5.6)$$

where $N$ is number of pulses and $\xi$ is the incubation coefficient.

It is necessary to consider the pulse overlap distance when analyzing scanned ablation experiments. Pulse overlap, denoted by $O_d$ refers to the fractional length of intersection between focus spots for consecutive pulses. It is related to the pulse repetition frequency $f$, focus spot diameter $d (=2w_0)$, and scan speed $s$ as

$$O_d = \left(1 - \frac{s}{df}\right). \quad (5.7)$$

The number of pulses $N$ incident on a single point along the scanned ablation path can be calculated by

$$N = \frac{d}{s/f} = \frac{1}{1-O_d}. \quad (5.8)$$

Ablated channel width $D_N$ for scanned ablation with pulse number $N$ can be calculated by combining Eqs. (5.4) and (5.6)

$$D_N = \sqrt{2w_0^2 \ln \left(\frac{F}{F_{th}(1)N^{\xi-1}}\right)}. \quad (5.9)$$
Furthermore, Eq. (5.8) is substituted into Eq. (5.9), results in

\[ D_N = \sqrt{2w_0^2 \ln \left( \frac{F}{F_{\text{th}}(1)(df'/s)^{2-1}} \right)} . \]  

(5.10)

Eq. (5.10) can be used to determine one unknown parameter such as incubation coefficient or predicted ablated width when the other terms are known.

Scanned ablation at different laser fluences and scanning speeds was conducted on the EGDMA surface and ablated microchannel widths were measured. Two different laser fluences (F=7.31 J cm\(^{-2}\) and 14.32 J cm\(^{-2}\)) at scanning speeds varying from 1.33 mm s\(^{-1}\) to 0.167 mm s\(^{-1}\) were used to create linear microgrooves on the EGDMA surface. Based on the scanning speeds, the corresponding pulse number (N) and pulse overlap (O\(_d\)) were calculated in accordance with Eqs. (5.7) and (5.8). The widths of the microgrooves D\(_N\) were measured from SEM images with examples being shown in Figure 5.7.
Figure 5.7 SEM image of linear grooves on the EGDMA sample produced by scanned ablation at different speeds and laser fluence of 7.31 J cm$^{-2}$.

A plot of the variation of microgroove width with scanning speed is displayed in Figure 5.8. At $F=7.31$ J cm$^{-2}$, the average channel width ranged from 4.93 µm to 4.23 µm as scanning speed varied from 0.167 mm s$^{-1}$ (corresponding to $N=106.2$) to 1.33 mm s$^{-1}$ ($N=13.3$). The lines in Figure 5.8 represent best-fit curves of Eq. (4.10) for laser fluences of $F=7.31$ and 14.32 J cm$^{-2}$. The previously determined values of $w_0=2.95$ µm and $F_{th}=3.76$ J cm$^{-2}$ were used and an incubation coefficient value of $\xi=0.83\pm0.03$ minimized the error of the two curves to the corresponding data. This incubation coefficient is dependent on the materials and the laser properties and current value is a typical range for various polymers from 0.9 to 0.6 reported by other researchers [9,24]. An incubation coefficient value of $\xi = 1$ corresponds to no incubation effect.
Figure 5.8 Incubation coefficient, $\xi$, was found to be $0.83 \pm 0.03$ by minimizing the error between Eq. (5.10) and linear scanned ablation channel widths at varying scanning speeds and different laser fluences $F=7.31$ and $14.62$ J cm$^{-2}$.

5.3.3 Microchannel fabrication on prepatterned EGDMA

With known single pulse ablation threshold and incubation coefficient, Eq. (5.10) may be used to predict microchannel width for varying average laser power (corresponding to laser fluence) and process scan speed (corresponding to laser pulse number, $N$). For initial trials, a laser power $P=3$ mW (corresponding to a fluence of 7.31 J cm$^{-2}$) and scanning speed of 0.33 mm s$^{-1}$ were assumed. The pulse number calculated
from Eq. (5.8) with \(w_0=2.95 \mu m\) is \(N=53.1\). Therefore, the ablation threshold is lowered from the single pulse value of \(3.76 \text{ J cm}^{-2}\) to an incubated threshold of \(F(53.1)=1.91 \text{ J cm}^{-2}\). Using this incubated ablation fluence, single pass ablation width can be estimated as 4.5 \(\mu m\). The total width of a channel ablated with 10 passes with lateral spacing of 10 \(\mu m\) is 13.4 \(\mu m\) as shown in Figure 5.9. This channel width was designed to be slightly larger than usual cell size (around 10 \(\mu m\)). Based on this analysis, one can easily predict a width of microchannel based on number of pass with ablation properties of EGDMA polymer. This is important step to determine number of pass for desired dimensions of microchannel before fabrication.

![Laser fluence distribution](image)

Figure 5.9 Laser fluence distribution at \(P=3\text{mW (F=7.31 J cm}^{-2}\)) with \(w_0=2.95 \mu m\). Using incubation coefficient \(\zeta=0.83\), incubated laser fluence \((N=53.1, \text{corresponding to scan speed of 0.33 mm/s})\) was calculated to be 1.91 J cm\(^{-2}\) and estimated microchannel width was 13.4 \(\mu m\) with 10 passes.
Figure 5.10(a) shows a SEM image of two inlet and outlet microchannels fabricated by femtosecond laser ablation on EGDMA polymer with 10 overlapping passes. Some re-deposited ablated material observed on the EGDMA polymer surface could be removed by ultrasonic cleaning. The magnified SEM image displayed in Figure 5.10(b) shows that the width of the microchannel is approximately 14 μm. It is found that there is only 0.6 μm difference between the experiment microchannel width and the width predicted based on incubated ablation threshold.
Figure 5.10 (a) Microchannel fabricated on EGDMA polymer. Laser power was 3mW ($F=7.31 \text{ J cm}^{-2}$) and scan speed was 0.33 mm/s ($N=53.1$ and $O_d=0.98$). Distance between two microchannels was 500 μm. (b) Magnified image of microchannel. Width of microchannel was measured to be around 14 μm.

Figure 5.11(a) shows an optical microscope image of prepatterned microwells and nanochannels produced by DCI as well as a 20 μm converging microchannel made by femtosecond laser ablation. The microwell diameter was 7 μm and the distance between microwells was 8.2 μm, which was also the length of the horizontal nanochannels that connected adjacent microwells. Figure 5.11(a) shows the 20 μm converging
microchannel fabricated by femtosecond laser ablation before filling with Rhodamine dye-water solution. After microchannel filling and allowing time for diffusion, the magnified fluorescence image displayed in Figure 5.11(b) was acquired to verify the extent of filling of the microwell/nanochannel array. The image verifies that the dye solution had diffused into the array near the converging channel (marked by white box), confirming that nanochannels (marked by white arrows) and microchannels were still open after laser machining of the microchannel. Figure 5.12 shows a confocal microscopic image of loaded Jurkat cells inside the microchannel made by direct write femtosecond laser ablation technique.
Figure 5.11 (a) The micro-converging channel (20 µm width) was fabricated by femtosecond laser ablation on the prepatterned microwells and nanochannels by DCI. (b) Magnified the fluorescence micrograph of micro channel/well and nano-channel filled by Rhodamine dye ($10^{-5}$ mol L$^{-1}$) to confirm that nano-channels (three specified by white arrows) are still open after post-fabrication of micro-channel by femtosecond laser.
Figure 5.12 Confocal microscopic image showing loaded Jurkat cells inside the femtosecond laser machined microchannel.

5.4 Conclusions

In summary, single pulse ablation and linear scanned ablation were conducted on an EGDMA sample. Single pulse ablation threshold was measured from single pulse ablation features and incubation coefficient was measured from the width of grooves machined by scanned ablation. The measured ablation properties were used to predict the width of microchannels machined by multiple side-by-side overlapping scans. Comparisons showed good agreement between the predictions and experiment results. A fluorescent dye flow test showed that fluid was able to pass from the laser-machined microscale filling channel into the nanoscale fluidic channels.
5.5 Acknowledgements

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5.6 References


Chapter 6

Vascular Wall Engineering via Femtosecond Laser Ablation

6.1 Introduction

Over 80 million people in the United States suffer from coronary vascular disease; within this group more than 176,000 patients annually undergo coronary artery bypass grafting [1]. The lack of healthy autografts, especially in the elderly, is a major obstacle to successful treatment [2]. Synthetic vascular grafts made of non-degradable polymers, such as Dacron or ePTFE, are commonly used for bypass grafting. Unfortunately, in vivo small (< 6 mm) diameter synthetic grafts often exhibit compliance mismatch, rapid plaque formation and occlusion of the lumen [3,4].

To provide a replacement vessel with biomimetic function and properties, tissue engineering strategies have been investigated such as cell sheets or cells cultured on biopolymers, such as collagen, to form engineered vessels[5,6,7]. A key component of these strategies is promoting proper cell organization and orientation within the vessel, especially in the tunica media (middle layer) of the vessel. Bioreactors with cyclic stretch [8], pulsatile perfusion [9], and microchannels [10,11] have all been successfully utilized to direct smooth muscle cell alignment. However, these methods can require up
to 5 months from initial cell culture to final vessel formation [12] which is incompatible with many clinical applications.

To more rapidly engineer microchannels within three-dimensional scaffolds for directed cell alignment, femtosecond (FS) laser ablation has been utilized [13,14] due to its flexibility and capabilities for precise material removal [15,16]. Femtosecond laser ablation is an effective method for three-dimensional structuring of nanofiber scaffolds made of biodegradable polymers while concurrently retaining fiber microstructure [17,18,19]. Electrospun polycaprolactone (PCL) tubes have demonstrated burst pressures above 2000 mm Hg [20]. In a prior study, direct-write femtosecond laser ablation was employed to create a series of microchannels (100 μm deep, 100 μm wide and 16 mm long) running the length of tri-layered 3-D electrospun scaffolds [21]. The tri-layer scaffold is comprised of a low porosity electrospun fiber layer creating a surface for endothelial cell attachment (tunica intima), a high porosity fiber layer containing femtosecond laser ablated microchannels that facilitate directed seeding, infiltration and organization of smooth muscle cells (tunica media) and a mechanically robust electrospun fiber layer to provide appropriate mechanical strengths (tunica adventitia). If designed correctly, this tri-layered structure containing microchannels can be formed without compromising mechanical overall properties [21]. However, the ability of these tri-layered scaffolds to promote cell attachment, viability and organization has not been investigated.

For the present work, a biodegradable polymer, polycaprolactone (PCL) was used as a tissue scaffold. Then, femtosecond laser was employed to create microchannels for beads or cell seeding. Flow within this microchannel was initially studied by injecting
fluorescent polystyrene beads through the microchannel. In addition, two dimensional (2-D) fluid modeling using computational fluidic dynamics (CFD) software was employed to estimate the fluid profile within the microchannel.

6.2 Materials and methods

6.2.1 Electrospun polycaprolactone

To fabricate the tunica intima equivalent that also serves to “seal in” cells seeded into the laser-ablated channel, a PCL solution was prepared by dissolving 14 wt % PCL (Sigma Aldrich, St. Louis, MO, M_w = 80,000) in acetone (Sigma-Aldrich, St. Louis, MO) heated to 60°C with continuous stirring to dissolve the polymer. Once cooled to room temperature, the solution was placed in a 60-cc plastic syringe (BD Luer-Lok, Franklin Lakes, NJ) equipped with a 20-gauge blunt tip needle (EFD, East Robinson, NJ). The solution was electrospun using a syringe pump (KD Scientific, Holliston, MA) at a flow rate of 16 mL h⁻¹ and a high voltage power supply (Glassman, High Bridge, NJ) set to a voltage difference of 24 kV. Electrospun fibers were collected onto standard glass microscope slides (Gold Seal, Ted Pella, Redding, CA) placed upon a 7.6 cm x 7.6 cm steel plate covered with aluminum foil at a distance of 20 cm from the needle tip for 10 min. After electrospinning, all samples were placed in a room temperature vacuum overnight to ensure that any residual solvents in the scaffold were eliminated.

6.2.2 Direct-write femtosecond laser ablation for microchannels

To fabricate microchannels on electrospun PCL nanofiber scaffolds, frequency doubled pulses from a mode-locked erbium-doped fiber laser intensified in a Ti:Sapphire
regenerative amplifier laser (CPA2161, Clark-MXR) were used. Laser wavelength was \(\lambda = 775\) nm. The maximum average output power of the laser was \(P_{av} = 2.5\) W, pulse duration was \(T_p = 150\) fs, pulse repetition frequency was \(f_p = 3\) kHz and collimated beam diameter was 5mm. The beam quality factors were \(M^2 = 1.2\) in the vertical Y direction and \(M^2 = 1.3\) in the horizontal X direction. Laser beam power was adjusted by a series of thin-film polarizing beam splitters and \(\frac{1}{2}\) waveplates. The attenuated laser beam was focused on the material with a 50x infinity corrected microscope objective lens having numerical aperture NA = 0.42 (M plan Apo NIR 50x, Mitutoyo). Attenuated average laser power was measured by a power meter (PM100, Thorlab) placed under the laser focusing lens. Sample motion and focus lens positioning was provided by a computer controlled motion system (MX80L, Parker) with 0.5\(\mu\)m resolution in the X, Y, and Z axes. The system was programmed to scan the electrospun nanofiber scaffold under the focused laser beam to fabricate patterns on its surface.

Next, linear grooves were produced by scanning the focused FS laser beam over the surface of electrospun acetone PCL nanofibers at various laser average powers. All ablation of linear grooves was performed at a scanning speed of \(s = 60\) mm/s. The average power of the FS laser was varied from 2mW to 20mW (the corresponding pulse energy range was from 0.67\(\mu\)J to 6.67\(\mu\)J). Ablated width was measured from SEM images and ablated depth was measured by moving z-location focusing lens with precise motion system with a step size of 0.5\(\mu\)m from top surface of nanofiber to bottom surface until it reached clear focused image.

After suitable ablation energy and scan speeds were determined from this initial study, direct-write FS laser ablation was used to fabricate single or 5 microchannels.
Focused FS laser beam was scanned over the surface of dogbone tensile test specimens prepared by previous step for single and 5 microchannels [21]. Dimensions of single and 5 microchannel were 100μm width and 100μm depth and 1600mm length, as depicted in Figure 6.1. The X - Y motion system scanned the sample with linear paths at a scanning speed of 0.33mm/s and laser pulse energy of 3.33μJ. An average laser power of 10mW (pulse energy of 3.33μJ) was selected based on the prior linear scan experiments. The programmed pattern used to machine 100μm width and depth consisted of 2 layers of 40 linear paths each. The spacing of each linear path was 2.5μm. The machining was done in two layers to produce microchannel with 100μm depth. After machining the first layer the focus position was adjusted downward by 50μm to ablate the second layer. Similarly, 5 microchannels were made at dogbone tensile test specimens with 100μm spacing of each channel. In the similar manner, single and five microchannels were made on acetone PCL nanofiber deposited on glass substrate for beads/cells flow test.
6.2.3 Fluorescent bead flow analysis of microchannels

Direct-write femtosecond laser ablation technique was applied to the electrospun fibers to create a microchannel 100 µm wide, 100 µm deep and 10 mm long. After laser machining, a plastic Teflon 25-gauge blunt needle tip (EFD, East Providence, RI) was cut at the end to create a taper that was then secured to the scaffold containing laser machined microchannels, taking care to position the plastic needle tip directly above one end of the microchannels. With the needle tip secure, the scaffolds received another layer of electrospun 14 wt % PCL in acetone with the same parameters used previously with
the exception of a 1 minute electrospinning time to create a thin (80 ± 12 μm) fibrous layer that permitted imaging but was thick enough to retain either beads or cells inside the microchannels during flow, as shown in Figure 6.2.

Fluorescent, Nile red polystyrene beads with a diameter of 20 μm (Spherotech, Lakeforest, IL) were diluted in deionized water to 0.2 wt. vol⁻¹ % and placed in a 5-cc plastic syringe (BD Luer-Lok, Franklin Lakes, NJ). A 33” extension with both female and male luer-lok (Medex, Monsey, NY) was used to attach the plastic needle tip to the syringe. Using a syringe pump, the fluorescent bead solution was flowed into the microchannels at the flow rate of 1 mL h⁻¹. An upright fluorescent microscope (Nikon Eclipse LV150, Melville, NY) was used to record bead motion down the microchannel at a recording rate of 50 frames per second. The video file was then extracted into time-lapse image files using Image J (NIH, USA).
Figure 6.2 Schematic for flowing 20 μm beads through the syringe into the microchannel (100 μm wide, 100 μm deep and 10 mm long). A thin layer (80 ± 12 μm) of electrospun PCL fibers lay on top of the microchannel.

6.2.4 Scanning electron microscopy

Scanning electron microscopy (FEI Quanta200 and FEI Sirion, Hillsboro, OR) was utilized both to examine microstructure of the laser machined microchannels as well as the post-seeded presence and distribution of beads and cells. All samples were adhered with conductive carbon tape to aluminum SEM sample mounts (SPI Supplies, West Chester, PA), sputter coated with ~100 angstroms gold (Pelco Model 3 Sputter Coater 9100, Clovis, CA) over a period of 60 s using an emission current of 15 mA and imaged using secondary electron mode at 5 - 12 kV.

6.3 Flow field modeling in the microchannel

Numerical simulation was carried out to model flow behavior in the microchannel ablated within the nanofiber-based porous scaffold. Modeling can produce predictions of
cellular deposition useful in future adaptations of this work in more extensive and complex microchannel geometries. Consider helically arranged microchannels running down the length of a hypothetical 2-cm long tubular construct 0.5 cm in diameter. If each microchannel occupies 0.1 cm tube length, the total microchannel circumference will be 314.2 cm and thus an understanding of how far cells can travel in the face of continuing media loss into the surrounding walls is of interest. However, in order to circumvent the complexity of modeling flow through the surrounding porous medium, a simplified 2-D duct flow model with constant flow out velocity boundary conditions on the walls was used to elucidate the experimental flow pattern. With the assumption of constant physical properties, the steady-state laminar incompressible flow is governed by the continuity and Navier-Stokes (N-S) equations in the forms of

\[ \nabla \cdot \mathbf{V} = 0 \]  
\[ \rho \mathbf{V} \cdot (\nabla \mathbf{V}) = -\nabla p + \mu \nabla^2 \mathbf{V} \]

where \( \mathbf{V} \) is the velocity field, \( \rho \) is the density and \( \mu \) is the dynamic viscosity.

The flow through a microchannel having a length of 10 mm and width of 100 μm (Figure 6.2) is simulated. The fluid was assumed to be distilled water and physical properties at room temperature were used for the computation. A uniform velocity profile with a magnitude of 2.78 cm s\(^{-1}\) obtained from experimental data was assigned at the inlet; the outlet was set at atmospheric pressure. A constant lateral velocity is used at the walls defined by:

\[ fV_{inlet}W = 2V_{wall}L \]

where \( f \) is the total fraction of volume flow rate flowing out from the wall, \( W \) and \( L \) are the width and the length of the microchannel and \( V_{inlet} \) and \( V_{wall} \) are the velocities at the
inlet and the wall, respectively (Figure 6.3). Therefore, $V_{\text{wall}}$ can be determined for different values of $f$. If $f = 1.0$, the entire flow originating from the inlet will flow through the wall and the velocity at the outlet will be zero. The computational domain was meshed with uniform grids 4 $\mu$m in size using GAMBIT preprocessor software. The governing equations were solved by FLUENT (ver. 6.3, ANSYS Inc., Canonsburg, PA) based on the finite volume algorithm.

![Figure 6.3](image.png)

Figure 6.3 A schematic of pressure driven flow with constant net volume flow loss ($f$) into lateral directions along the microchannel.

### 6.4 Results

#### 6.4.1 Electrospun PCL nanofiber scaffolds

Utilizing the conditions given in the materials and methods section, the sheet thicknesses produced were: (1) 14 wt % PCL dissolved in acetone, $358 \pm 33.5$ $\mu$m.
Figure 6.4(a) and (b) show SEM images of acetone PCL nanofiber deposited on glass substrate.

![SEM images of acetone PCL nanofiber](image)

Figure 6.4 SEM images of electrospun acetone nanofiber (a) and (b), electrospun DCM nanofiber (c) and (d)

### 6.4.2 Direct-write femtosecond laser ablation for microchannel fabrication

SEM images of FS laser ablated grooves made by linear scanned ablation at various laser pulse energies are shown Figure 6.5. The width and depth of the grooves were analyzed in more detail to determine suitable process parameters for machining of microchannels. Average, maximum and minimum widths and depths of grooves in ablated electrospun acetone PCL nanofiber at different pulse energies are plotted in Figure 6.6(a) and (b). The average maximum groove widths increased from about 11.5μm
to 118μm as pulse energy increased from 6.67μJ to 0.5μJ. Groove depths increased from 10μm to 63.5μm as pulse energy was increased from 0.67μJ to 6.67μJ. The specific energy $H$ of the ablation plotted in Figure 6.6(c) is the optical energy required to ablate material during machining of grooves by a scanned laser focus spot. It was calculated from the average laser power $P_{av}$, average groove width $w$, average groove depth $d$ and linear scan speed $s$ as

$$H = \frac{P_{av}}{wds}$$

(6.4)
Figure 6.5 Femtosecond laser scanned ablation on acetone PCL nanofiber with different laser pulse energies at a scan speed of 60 mm/s.
Figure 6.6 Sizes and specific energy of features ablated in electrospun acetone PCL nanofiber using a 0.42 NA focusing objective and scan speed of 60 mm/s at various pulse energies (a): width, (b): depth, and (c): specific energy.
Next, the femtosecond laser ablation conditions utilized produced microchannels 100 μm wide, 100 μm deep and 16 mm long, as shown in Figure 6.7. Also, Figure 6.8 shows a single microchannel with dimensions of 100 μm wide, 100 μm deep and 10 mm long for beads/cells flow test purpose. It is seen that femtosecond laser machining successfully produced a microchannel in the PCL scaffolds without substantially altering the microstructure of the fibers. No significant melting of fibers either in or surrounding the microchannels themselves was observed. Additionally, the margins appear to be uniform down the length of the microchannels.

Figure 6.7 SEM image of a single microchannel machined on acetone PCL nanofiber, (b) 5 microchannels produced on acetone nanofiber for mechanical tensile test. The width was 100μm and spacing between each microchannel was 100μm.
Figure 6.8 SEM of femtosecond laser ablated 100 μm x 100 μm x 10 mm microchannels in electrospun PCL scaffolds for beads/cells flow test.

6.4.3 Bead/ cell seeding in the microchannels

Flow tests using the 20 μm diameter fluorescent beads were conducted using the 100 μm by 100 μm by 10 mm length microchannels. The thin electrospun layer capping the microchannels did not appear to obstruct bead flow during the experiment. Figure 6.9 shows that the fluorescent beads stayed within the microchannels and successfully flowed down the length of the microchannels without any apparent buildup or obstruction. The velocity of the beads in the microchannel was 0.6 cm s⁻¹ as determined from the time-lapse images (Figure 6.10). This velocity was measured near the edge of a microchannel.
Figure 6.9 Fluorescent beads stay within the microchannels as shown in (a) SEM and (b) fluorescence microscopy.

Figure 6.10 Time lapsed images for fluorescent beads flow test. Bead flow velocity was measured to be $0.6 \text{ cm s}^{-1}$ between 8 and 9mm of the channel length at flow rate of 1 mL H$^{-1}$. 
6.4.4 Flow field in microchannels

Pressure driven flow into the microchannels was simulated using CFD software. To study the effect of fluid loss, the flow patterns of different values of \( f \) ranging from 0 to 1 with increments of 0.1 were studied. As shown in Figure 6.11, the velocity profiles along the microchannels were plotted for \( f = 0.5, 0.8 \) and 1. It is shown that the flow velocity is reduced toward the end of the microchannel due to the fluid loss from the wall. In Figure 6.12, the maximum velocity \( (u_{\text{max}}) \) in the center of the microchannel is plotted under different degrees of fluid loss \( (f) \) into the walls. The Reynolds number \( (Re) \), which is the ratio of inertial forces to viscous forces, is calculated based on

\[
Re = \frac{\rho VL}{\mu}
\]  

(6.5)

where, \( \rho \) is a density of the fluid, \( V \) is the mean velocity of fluid, \( L \) is a characteristic length, and \( \mu \) is the viscosity of the fluid, respectively. The small value of \( Re \) number is calculated to be \( 2.1641 \times 10^{-7} \) based on the width of the microchannel. Since flow is in the micron scale, the viscous force is dominant. Therefore, the entrance effect is minimal and the fluid is quickly accelerated to the peak value in a short distance as seen in Figure 6.12. After reaching a peak value, the maximum velocity decreases linearly for different \( f \) values. As \( f \) becomes larger, the velocity decrease becomes greater.
Figure 6.11 Comparison of fluid profile with different fraction of fluid volume loss ($f=0.5$, 0.8 and 1) along the microchannel.
Figure 6.12 Comparison of maximum fluid velocity ($u_{\text{max}}$) at the center of microchannel width with different fraction of fluid volume loss from $f=0$ to 1 into nanofiber walls.

### 6.5 Discussion

For grooves produced by scanned ablation with a laser pulse energy of $6.67\mu\text{J}$, the average maximum ablated groove width was measured to be $118\ \mu\text{m}$. At pulse energies below $0.67\text{J}$, the measurements of lateral dimensions of single-pulse ablation volumes were more variable because of the effects of the mesh porosity of acetone PCL nanofiber limited the measurement resolution. For this reason, the ablation width and depth curves in Figure 6.6(a) and (b) were not continued below $15\ \mu\text{m}$. Even this minimum measurable ablation groove width is much larger than the optical focus spot diameter of $3.34\ \mu\text{m}$ for
50x focusing lens obtained from the previous PCL/gelatin nanofiber case [19]. Also, the measured groove depths were similarly large in comparison to the calculated optical depth of focus of 3.46 μm. The laser focus spot diameter, pulse repetition rate and scanning speeds used for scanned ablation produced a focus spot overlap of -3, meaning that centers of the focus spot for consecutive laser pulses were separated by 3 Gaussian focus spot diameter (approximately 3.34 μm) along the scan path. In spite of this large separation of successive optical pulse locations, the scanned ablation produced mostly continuous grooves. Figure 6.6(c) shows the specific energy of the ablation of acetone PCL nanofiber. The average specific energy for scanned FS laser ablation was less than 0.2 J/mm³ for all pulse energies. The pulse energy used for subsequent microchannel machining (E=3.33 μJ) was selected as a compromise which yielded acceptable feature resolution (lateral resolution of approximately 40 μm and a depth resolution of approximately 30 μm) and minimal fiber melting at some expense of material removal efficiency.

Femtosecond laser ablation is a unique tool allowing precision machining of polymer-based scaffolds, producing complex flow channel networks in electrospun scaffolds while preserving the underlying nanofiber structure [10]. By creating two- and three-dimensional patterns, favorable cell-cell interactions can be achieved. To best enable this manufacturing focus, we must develop the scientific knowledge needed to design and fabricate scaffolds with fully reproducible surface features at resolutions approaching approximately 10 μm. The diameters and depths of desired helical passages and cavities are large compared to the diameter and the depth of material removed by a single pulse. Thus ablation must be incremental – as it is in this work – with each
individual layer being ‘machined’ by pulses of laser energy that intersect (‘overlap’) in the axial and circumferential motion directions as individual laser pulses (typically 20μm - 50μm in diameter and depth) are superimposed to produce the overall cavity. In the eventual design, helical flow channels will circle around the tube and have cross sectional sizes of approximately 100 μm by 100μm. Knowledge of the variation of strength in the presence of these microchannels is especially important because it can determine the size of the channels within the “tunica intima layer.” The process relationships derived during this initial work provide a starting point for this process study. Our final goal is to achieve a tubular formation which is a helical microchannel running down the length of a cylindrical tissue engineered vessel, as illustrated in Figure 6.13. This microchannel would sit within the “tunica media” layer where seeded smooth muscle cells can infiltrate the microchannel and align in the appropriate direction necessary for the appropriate in vivo function.
Figure 6.13 Schematic of the proposed trilayer structure with femtosecond laser ablated microchannels in the middle tunica media to mediate alignment of smooth muscle cells.

In the current study, femtosecond laser ablation created 100 µm x 100 µm x 10 mm channels in 3-D PCL scaffolds in a single step process with precise material removal and minimal alterations to fiber morphology [17,19]. Femtosecond laser ablation is a unique tool allowing precision machining of polymer-based tissue scaffolds, producing complex flow channel networks in electrospun scaffolds while preserving the underlying nanofiber structure. Femtosecond lasers produce short duration (FWHM~150fs) pulses which, when focused to a spot size of several microns, provide large irradiance (>TW/cm2), fluence and photon flux. As a result, the beam-material interaction is non-linear and energy absorption occurs only when the fluence is above a distinct material
dependent threshold. When used for ablation, the femtosecond laser can remove material within a region smaller than the spot size; microscale resolution is enhanced vis-à-vis nanosecond lasers. Using this ability to use this to form channels in a 3D scaffold is a large step forward in the fabrication of more complex tissue engineering structures. The precision and reproducibility obtained by femtosecond laser ablation allows for a range of patterns to be machined into polymer scaffolds for tissue engineering applications that cannot be matched by other techniques.

During the seeding experiments, the thin acetone-derived fiber layer electrospun over the microchannels successfully covered them ensuring that neither beads nor cells could leak out. Because electrospinning is not a conformal technique, no fibers were deposited inside the microchannels due in part to their relatively small size. However, this bead- and cell-impermeable layer was thin enough to allow direct imaging of as-deposited beads and cells.

Fluorescent bead seeding allowed imaging of bead motion inside the microchannels due to their relatively large size (20 μm diameter), fluorescent intensity and the slow flow rate (1 mL h⁻¹). Their size also kept the beads inside the ablated microchannels and prevented infiltration out into the surrounding scaffold. The velocity of the beads in the microchannel was found to be 0.6 cm s⁻¹ using time lapsed images from Figure 6.10. Additionally, the mean input velocity was calculated by dividing the volumetric flow rate by the cross sectional area of microchannel to get 2.78 cm s⁻¹. If there is no fluid loss, theoretically a fully developed parabolic velocity profile should be obtained in which the maximum velocity in the center of the microchannel will be 1.5 times the mean velocity [22], resulting in $u_{\text{max}} = 4.125$ cm s⁻¹. However, it was observed
that the fluid diffuses in the lateral directions along the microchannel during flow because of the porous microstructure inherent to electrospun scaffolds (Figure 6.8). Fluid is being transported into the nanofiber walls via a combination of pressure gradients and capillary action. This explains why the calculated experimental velocity was less than the calculated analytical velocity in the microchannel. In Figure 6.11 and Figure 6.12, the fluid velocity linearly decreases as the front moves toward the end of the microchannel. When the fluid loss $f$ is equal to 1, fluid velocity is zero near end of the microchannel. To establish the proper $f$ value, the calculated bead velocity was compared with the numerical simulation results for different values of $f$. From the experiment, bead flow velocity was 0.6 cm s$^{-1}$ at between 8 and 9 mm of the microchannel length. As a result, the proper $f$ range is from 0.9 to 1 (Figure 6.12). The value of $f$ could be related to the porosity of the PCL nanofiber. Since electrospun PCL nanofiber has a high porosity, more than 90% [23], more fluid losses are expected through the porous walls in the microchannel. This result is consistent with that of other studies [24].

6.6 Conclusions

Femtosecond laser ablation successfully produced these microchannels in the scaffolds without substantially altering the ~700 nm diameter fibers. The dimensions chosen were 100 μm wide, 100 μm deep and 10 mm long. The ability of such laser machined microchannels to direct bead seeding was evaluated. Flow within these microchannels was studied by injecting fluorescent polystyrene bead solutions. Direct measurement of bead motion yielded an inlet velocity of 2.78 cm s$^{-1}$. This was used to model two-dimensional flow using computational fluid dynamics to estimate flow.
profiles within the microchannel. The concept of “vascular wall engineering” producing intricate bead seeding through microchannels produced via femtosecond laser ablation was validated.

### 6.7 Acknowledgements

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6.8 References


Chapter 7

On-going research for Automated Cell to Biomolecule Analysis (ACBA) Nanofactory System

7.1 Introduction

Nanoscale Science and Engineering Center (NSEC) Center for Affordable Nanoengineering of Polymeric Biomedical Devices (CANPBD) has been funded by National Science Foundation (NSF) to develop polymer based devices by employing nanotechnology and biotechnology for high precision cell separation, cell manipulation and biomolecule detection. It is called Automated to Cell Biomolecule Analysis (ACBA). To capture and identify circulating tumor cells (CTCs) for early cancer discovery is a main goal for. This is still on-going collaboration researches among NSEC faculty members.

As shown in Figure 7.1, there are total three stages in ACBA platform. The first stage is cell separation stage. Next one is cell manipulation stage. The last stage is bio-detection stage. There are two sub-groups in ACBA platforms, based on cell manipulation techniques; optical tweezers and magnetic tweezers. Currently, we are currently involved in Nanofiber-based Cell Separation group for the 1st stage, and antibody coated nanoelectroporation for bio detection at the 3rd stage.
To achieve our center goal, micro/nano scale material processes are necessary for required device fabrications. As a core member of nonmanufacturing group in NSEC, we utilized one of the non-clean room techniques that achieve this is laser ablation, specifically femtosecond laser ablation. Laser ablation by pulses with duration on the sub-picosecond or femtosecond time scales can remove materials with lower residual thermal effect, and the accuracy and quality of device is often superior to conventional longer-pulse lasers. The process is developed for use in a non-clean room environment that reduces the cost of final product allowing affordable device fabrication. Also, it provides a convenient, economical and flexible way to fabricate programmable 3-dimensional patterns by varying the beam scanning speed during ablation as well as laser
pulse energy. In addition, femtosecond laser ablation technique can be used as a primary or a post process.

Micropatterning – and pattern removal – of electrospun biodegradable nanofiber is difficult because of its high porosity. Although UV photolithography was recently reported for micropatterning of electrospun biocompatible polymer for tissue engineering applications, but this still requires use of a photomask and several hours to degrade the nanofiber scaffold [1]. Femtosecond laser ablation is a relatively faster, more affordable method suited to many different materials and can be used for biomedical applications. Our work used femtosecond laser ablation technique to fabricate biomedical devices for optical tweezers-cell array nanoelectroporation and nanofibers based cell separation for ACBA platform. We demonstrated femtosecond laser material process for polyvinyliden fluoride (PVDF) polymer and electrospun poly(ε-caprolactone) (PCL) nanofiber scaffolds for ABCA platforms.

7.2 Nanofibers based Cell Separation for 1st stage

With collaboration with Dr. Lannutti (MSE) and Dr. Hansford (BME) groups, nanofibers based cell separation device was designed and fabricated, as shown in Figure 7.2. For nanofiber-based cell separation, relatively short cell migration times are necessary to produce biomedical readouts in the shortest amount of time. Several methods, including chemotactic stimuli, or alternation of chemical or physical property of nanofiber can be used to enhance the cell migration. Another way to reduce the cell migration time is to create a relatively short physical travel distance for cell movement. Since electrospinning cannot control the length of nanofiber deposited during the process, post processing is required. Femtosecond laser ablation can easily alter the highly
randomized product of electrospinning and can precisely cut the aligned nanofiber for desired length control for nanofiber-based cell separation.

Figure 7.2 Schematic of nanofibers based cell separation for 1st stage.

Such defined cut nanofiber interfaces are critical to the use of either optical or magnetic tweezers to remove cells arriving at a well-defined interface. A thin layer of the aligned PCL nanofiber electrospun onto an ITO-coated glass substrate was provided by Dr. Lannutti’s group, as shown in Figure 7.3(a). Femtosecond laser ablation was then used to cut the aligned PCL on the glass substrate for subsequent cell manipulation. 50x (NA=0.42) focus optic was used to cut the nanofibers. The average laser power was 1mW (correspond pulse energy of 0.33μJ). Figure 7.3(b) shows the optical images of cleanly ‘cut’ aligned PCL nanofiber. Clearly, the nanofiber structure was maintained during this
process even though PCL has a relatively low $T_m$ ($\sim$60°C) [2]. Femtosecond laser ablation plays a very important role in the Nanofiber Racetrack ACBA by delimiting the nanofiber lengths on both sides of the array. No other technique we are aware of can cleanly ‘cut’ the nanofiber with this level of precision while simultaneously preserving the nanoscale.

![Figure 7.3 PCL nanofiber deposition on the glass substrate (a); PCL nanofiber cutting by femtosecond laser ablation (b).](image)

Similarly, a thin layer of aligned PCL nanofibers were deposited on silicon substrate possessing magnetic disks used for magnetic tweezer manipulation of magnetic particles attached to cells, as shown in Figure 7.4. Figure 7.5 depicts an SEM image of aligned PCL nanofiber cut in-between vertically-oriented columns of magnetic disks patterned onto a silicon wafer. The average laser power was 1 mW (correspond pulse...
energy of 0.33 μJ) and a scan speed of 120 mm/min was used for cutting of PCL nanofibers. It was observed that there was ablation of both the underlying silicon substrate and the PCL nanofiber. This is due to the laser ablation threshold fluence difference for silicon and PCL material. The ablation threshold fluence for silicon material [3] is $F_{th}(\text{silicon})=0.26 \text{ J/cm}^2$, while bulk PCL has a relatively higher ablation threshold than silicon. We tested magnetic particles movement on the silicon sample. In collaboration with the group led by Dr. Sooryakumar (Physics), we found that there was no significant effect on the movement of magnetic particles under the influence of the magnetic tweezer.

![Image](image.png)

Figure 7.4 (a) Optical image, showing aligned PCL nanofiber deposited on silicon substrate with magnetic disks. (b) Magnified SEM image.
Figure 7.5 SEM image of PCL nanofiber cutting by femtosecond laser ablation. Laser average power, $P=2\text{mW}$ and scan speed of $S=120\text{mm/min}$ were used.
7.3 PVDF membrane drilling by FS laser for nanoelectroporation hole array at 3\textsuperscript{rd} stage

The 3\textsuperscript{rd} stage at ACBA platform is a bio-detection. High through output of gene delivery idea was proposed from Dr. James Lee (ChBE) for this bio analysis purpose. Using electroporation technique, foreign genes or other molecules can be delivered into cells. This is one of the important methods in the biotechnology. However, conventional bulk electroporation techniques have a limitation on targeting specific cells. If specific cells are captured from a solution, this process could be more selective and valuable for bio-detection or biomolecule analysis. Collaboration with Dr. James Lee’s group members, we used a femtosecond laser drilling technique to fabricate the cell trapping membrane. Figure 7.6 shows a schematic of PVDF membrane drilling by femtosecond laser ablation for electroporation technique. In brief, a fabrication procedure of the cell captured device is described. Thin PVDF membrane can be prepared on PDMS or glass substrate by spin coating technique. Next, array of gold disk can be deposited on PVDF membrane using photolithography. After then, this sample will be covered with another PDMS and flipped over. Top side of PDMS will be removed and then transferred to the translation X, Y motion stages for femtosecond laser drilling process. Then, the drilled membrane will be mounted on cell culture insert. Anti-body will be coated on the array of gold disk to capture the individual cells. Finally, electroporation will perform for delivery gene into cells.
Figure 7.6 Schematic of PVDF membrane drilling by FS laser for nanoelectroporation, 3rd stage of ACBA platform.

Via the advantages of direct-write femtosecond laser ablation, this technique was used to produce an array of nanopore in spin coated PVDF on a glass or PDMS substrate. The PVDF sample was prepared from Dr. Hansford group. The thickness of PVDF membranes was found to be measured approximately 5μm. First of all, single pulse laser ablation test was conducted on the PVDF membrane with varying laser pulse energies from 0.67μJ to 0.066μJ. For the experiment, 100x (NA=0.5) laser focus lens was employed. Ablated diameters from top and bottom side were measured from scanning electron micrographs (SEM). Figure 7.7 shows SEM images for different laser pulse
energies. It was observed that there was no thermal damage around the ablated holes. Figure 7.8 plots the measured ablated diameter vs. different laser pulse energies. The ablated diameters varied from 0.9 to 4.7 μm as laser pulse energies from 66.6 nJ to 4.67 μJ. Figure 7.7(e-f) show SEM images, depicting the smallest pore produced by single pulse ablation with a laser average power of 0.2 mW (corresponding pulse energy of 66.6 nJ). The measured average ablated diameter was around 900nm at the top and 400 nm at the bottom of the membrane.
Figure 7.7 Example of SEM images for single pulse laser ablation test on PVDF membrane with varying laser pulse energies from 0.33µJ to 0.066µJ (a)-(d). Magnified SEM images (e) and (f) at the laser pulse energy of 0.066µJ [white box at figure (d)]. 100x (NA=0.5) objective lens was used.
Figure 7.8 Single pulse ablation experiment on PVDF membrane with varying femtosecond laser pulse energies and 100x (NA=0.5) objective lens.

Based on the measurement of ablated diameter at top side, effective Gaussian beam radius and ablation threshold fluence were found. The effective radius of the laser focus spot needed for fluence calculations can be calculated from the slope of a plot of experimental ablation diameter-squared versus pulse energy data. Ablated diameters $D^2$ are plotted against pulse energy in Figure 7.9(a). Effective Gaussian beam radius $w_0$ was found to be 1.14 $\mu$m from the slope the curve. Laser fluence was next calculated using the effective laser focus spot diameter and ablation diameter-squared versus laser fluence.
was plotted as shown in Figure 7.9(b). Single pulse ablation threshold fluence $F_{th}$ was calculated by extrapolating to zero ablation spot diameter, resulting in $F_{th} = 2.24 \text{ J/cm}^2$ for PVDF membrane.

Figure 7.9 (a) Squared ablated diameter versus laser pulse energy for single-pulse ablation tests. Effective Gaussian radius ($w_0$) was found to be 1.14 µm from the slope of the curve. (b) Single-pulse ablation threshold was calculated by extrapolating the ablated diameter$^2$ versus laser fluence curve to zero diameter. Ablation threshold fluence was calculated as $F_{th} = 2.24 \text{ J cm}^{-2}$ for PVDF membrane.

For comparison with other polymers, this ablation threshold fluence is slightly smaller than poly(methyl methacrylate) (PMMA), such as 2.6 J/cm² [4]. This difference results from the optical bandgap difference for other polymers. Kruger and Kautek [4] studied ablation threshold fluence for various polymers, such as polyimide (PI), poly(ethylene terephthalate) (PET), and PMMA using 800nm wavelength and 150 fs laser. Single pulse ablation threshold fluences for PI, PET, and PMMA were found 1.0, 1.8, and 2.6 J/cm²,
respectively. Optical bandgap was also found from previous literatures, such as 1.98 eV for PI [5], 3.8 eV for PET [6], and 5.75-6.07 eV for PMMA [7], respectively. For PVDF, optical bandgap is 5.35 eV [8]. Based on the optical bandgap difference, ablation threshold fluence (2.24 J/cm²) for PVDF should be larger than PET (1.8 J/cm²) and smaller than PMMA (2.6 J/cm²), and our works is well matched. The reason is that an increasing optical bandgap suggests a multiphoton absorption. That is, the material, which has the higher bandgap, needs a larger number of photons to overcome the gap in a one-step multiphoton absorption process [4]. Therefore, ablation threshold fluence for PMMA is greater than the threshold of PI, PET, or PVDF. Similar trend was found for other dielectric materials, such as fused silica (9eV) and barium aluminum borosilicate glass (BBS) (4eV) by Lenzner et al [9].

Next, an average laser power was further down to 0.07mW (corresponding pulse energy of 23.3nJ), which is lower than the minimum pulse energy of 66.6nJ from previous single pulse laser ablation test. Then, we applied multiple pulses overlapped on the same location. Due to incubation effect, PVDF membrane can be ablated below the minimum laser pulse energy. Laser dwell time was initially set to be 4 milliseconds which is a maximum mechanical shutter on/off response time. This laser on time corresponds to 12 pulses overlapped in the same spot. The magnified SEM image for PVDF drilled by femtosecond laser is shown in Figure 7.10. The measured ablated diameter was found to be 630nm at top surface. The ablated diameter at bottom should be measured for next step. This is still on-going project. For future work, it is required to find incubation coefficient for PVDF membrane. Therefore, we can find incubated laser fluence for this polymer with different number of pulses. Also, it is needed to find
optimized laser process parameters, such as laser pulse energy and laser dwell time to achieve the smallest pore size which is through the membrane thickness in z-direction. Once we find the minimum pore size with the optimum process parameters, we can produce a nanoscale array of holes can be produced.

Later on, this array of nanopore on PVDF membrane will be coated by anti-body from Dr. James Lee’s group for antibody coated nanopore array of cell capture device and will be used for nanoelectroporation (NEP) at detection stage of ABCA platform.

Figure 7.10 SEM image of PVDF membrane drilled by femtosecond laser ablation. Laser power was 0.07mW and laser dwell time was 4ms, corresponding 12 number of pulses overlapped.
7.3 Summary

In this brief, we demonstrated very clean cut for aligned electrospun PCL nanofiber for nanofiber-based cell separation platform using femtosecond laser ablation. Femtosecond laser ablation technique was also used to produce array of holes on PVDF membrane for nanoelectroporation at 3rd stage. Femtosecond laser ablation is a unique and fast material process technique for high aspect ratio features.

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7.5 References


Chapter 8

Conclusions

In research described in this dissertation, it is demonstrated that femtosecond laser provides a unique technique for micromachining both solid and porous materials in ambient, non-cleanroom conditions. High peak intensity enables ablation with minimal thermal effect on the surround material, consequently resulting in precise machining of three dimensional geometries to fabricate a number of microscale devices. Femtosecond laser ablation characteristics, such as laser ablation threshold fluence, incubation coefficient, were experimentally studied for various materials. Calculation with easily measured material ablation properties enables precision fabrication. Microscale pillars were fabricated on the bovine cortical bone and microchannels were made on the pre-patterned EGDMA polymer substrate. Femtosecond laser micromachining was effectively used to create microwells and microchannels on electrospun polycaprolactone and polycaprolactone + gelatin fiber materials. From these studies, it can be concluded that femtosecond laser micromachining is a versatile and promising technique for rapid fabrication of a wide range of microscale devices and features.
Future work is required for femtosecond laser micro/nano scale machining process. It is necessary to reduce machining process time for a fabrication standpoint. If a high pulse repetition rate (PRR) femtosecond laser system, more than megahertz (MHz), can be used, it is possible to increase a material ablation rate, consequently, further increasing scan speed. Next, beam shaping for femtosecond laser, such as flat-top shape, can be employed to improve the quality of laser machining. This flat-top beam shape has an intensity profile that is flat. Since most of femtosecond laser systems have a Gaussian distribution, the ablated shape is more likely a conical shape. If more straight vertical shape in z-direction is required, flat-top shape can be used for micromachining process. Additionally, many new materials have been developed these days for many different applications. Therefore, it has to keep studying femtosecond laser and the new material interaction.
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