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Femtosecond Laser Ablation Enhances Cell Infiltration into Three-Dimensional Electrospun Scaffolds

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Abstract

Electrospun scaffolds are used extensively in tissue engineering applications since they offer a cell-friendly microenvironment. However, one major limitation is the dense fibers, small pore size and consequently poor cell infiltration. Here, we employ a femtosecond (FS) laser system to ablate and create microscale features on electrospun poly(L-lactide) (PLLA) nanofibrous scaffolds. Upon determining the ablation parameters, we pattern structured holes of varying diameters of 50, 100, and 200 μm and spacing of 50 and 200 μm between adjacent holes on the scaffolds. The elastic moduli of ablated scaffolds decrease with the decrease of spacing and the increase of hole size. Cells seeded on the laser-ablated scaffolds exhibit different morphology but similar proliferation rate when compared with control (non-ablated) scaffold. Furthermore, animal studies indicate that ablated scaffolds facilitate endothelial cell ingrowth as well as drastically increase M2 macrophage and overall cell infiltration. These findings demonstrate that FS laser ablation can be used to increase cell infiltration into nanofibrous scaffolds. Laser ablation not only can create desired features in micrometer length scale but also presents a new approach in the fabrication of three-dimensional porous constructs for tissue engineering.

Keywords

Electrospinning; nanofibrous scaffolds; pore size; cell infiltration; femtosecond laser ablation

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1. Introduction

Biocompatible three-dimensional scaffolds play a pivotal role in tissue engineering. These scaffolds are fabricated with controlled mechanical and biological properties so that they are able to support new tissue formation while providing physical and chemical cues that promote various cellular processes, including proliferation, migration, and differentiation [1]. Specifically, the architecture of tissue-engineered scaffolds is designed to closely mimic the organized structure and biological function of native extracellular matrix (ECM). Thus, a favorable ECM-like microenvironment for cells is crucial to achieve the desirable scaffold integration as well as cell-material interactions and tissue ingrowth.

Electrospinning is a popular technique used to fabricate tissue-engineered scaffolds [2-5]. It is a consistent, versatile method that produces nonwoven, three-dimensional fiber structures with controllable fiber diameter [6-8]. Electrospun scaffolds have great potential in that their structures, especially in nanoscale network, not only closely resemble natural ECM but also exhibit high surface-area-to-volume ratio favorable for attachment of cells and bioactive molecules onto fiber surfaces [9]. However, one main issue that limits their utilization is their small pore size. Such small pore size of electrospun scaffolds results from the dense network of fibers, which hinders cell infiltration and ultimately reduces their use in replacing large tissues that require ample vascularization and nutrient diffusion. Various techniques to overcome this shortcoming have been investigated, including the incorporation of sacrificial fibers and porogens [10, 11], modification of fiber diameter [12], and postprocessing by photopatterning [13] or ultraviolet radiation treatment [14] to increase pore size and overall porosity.

Although laser machining is an attractive approach for many biomedical applications, a major concern with using this method to process biomaterial scaffolds is the potential for thermal effects, for example, induced by nanosecond laser. However, ultrafast laser is considered a promising tool to rapidly process and create complex structures on electrospun scaffolds. For example, although a few studies have investigated the use of femtosecond (FS) laser to ablate electrospun fibrous scaffolds since its ultrashort and high irradiance pulses produce clean ablation with minimal thermal and physical stress [15, 16], they focus primarily on surface ablations, such as grooves and microwells, which did not address the pore size issue of electrospun scaffolds, and there was limited biological characterization. In addition, key features that make FS laser processing superior over other laser machining processing, such as nanosecond or picosecond laser, lie primarily in its very rapid creation of vapor and plasma phases that lead to negligible heat conduction and the absence of liquid phase [17]. Thus, FS laser possesses the ability for precise material processing with clean surface and reduced thermal damage, whereas UV nanosecond laser ablation leads to redeposition of the material and results in melted morphology and decreased porosity [18, 19]. In fact, the level of precision in which the location and depth of well-defined ablation can be carefully regulated further facilitates the process.

Here, we explore the potential of FS laser ablation to create desired microscale topographical features and increase both pore size and bulk porosity of electrospun PLLA nanofibrous scaffolds. Using an 800 nm, 100 femtosecond (fs) laser to ablate the scaffolds, we determine optimal ablation parameters, characterize the process for patterning holes of varying diameters (50 μm , 100 μm , and 200 μm) and densities (50- μm and 200- μm spacing between adjacent holes), and compare the structure and mechanical property of the scaffolds. Furthermore, we assess the biocompatibility and biological performance of these ablated scaffolds to determine whether different extent of porosity from the patterned holes affect cell adhesion, morphology, and proliferation *in vitro*, along with cell infiltration and host response *in vivo*.

2. Materials and methods

2.1 Fabrication of PLLA nanofibrous scaffolds

We used eletrospinning, as described previously with minor modifications [20, 21], to fabricate nanofibrous scaffolds from biodegradable poly(L-lactide) (PLLA) (1.09 dL/g inherent viscosity) (Lactel Absorbable Polymers, Pelham, AL). The PLLA (19% w/v) solution was prepared by dissolving PLLA pellets in hexafluoroisopropanol (HFIP) solvent via sonication for 30 minutes until all of the PLLA pellets were completely dissolved. To deliver the polymer solution, a programmable pump was used along with a 5-ml syringe filled with PLLA that was fitted with flexible silicon tubing connected to 1.5" long stainless steel 23G dispensing needles. A high-voltage generator was used to apply a 12 kV voltage to the needle. PLLA fibers with diameters ranging from 200 nm to 3 μ m were ejected from the charged needle towards the surface of a grounded collector to form a nonwoven nanofibrous membrane. Specifically, the PLLA solution was delivered at a flow rate of 1 ml/hr from a gap distance (distance between needle tip and collecting drum) of 8.5 cm. For this study, a random orientation of nanofibers was achieved by using a low speed of rotation (200 rpm) for the collecting drum. Electrospinning continued until the scaffolds reached an approximate thickness of 130-200 μ m based on measurements with a thickness gauge (Mitutoyo America, Aurora, IL). The electrospun sheets were then placed in a vacuum overnight to remove any residual HFIP.

2.2 Femtosecond laser ablation and characterization of ablated nanofibrous scaffolds

The ablation characteristics of PLLA scaffold were evaluated to determine the optimal parameters for creating the desired ablations. We used a Ti:Sapphire amplified laser system (Spitfire, Spectra physics, Irvine, CA) and 5x objective lens with numerical aperture N.A. = 0.14 (M Plan Apo, Mitutoyo, Barrington, NJ) to perform FS laser ablation. First, using single pulses of the FS laser, we varied the laser energy and observed the corresponding change in ablation pore size. The pulse energy was adjusted with a half wave plate ($\lambda/2$) and a polarizing beam splitter. Samples were loaded on motorized stages (ANT130, Aerotech Inc., Pittsburgh, PA) with a synchronized laser trigger system controlled by a personal computer. Subsequently, using constant laser energy (35 μ J), we varied the pulse number to determine the resulting change in depth of the ablation with increasing pulse number. The ablated scaffolds were examined and imaged from both top and cross-sectional views to compare changes in pore size and depth, respectively, with Hitachi TM-1000 scanning electron microscope (SEM) (Hitachi, Pleasanton, CA). For the samples used in *in vitro* and *in vivo* studies, through-holes were fabricated on the PLLA scaffolds by circular and linear motion of the motorized stages with 1 mm/s speed with fixed laser energy (35 μ J) and pulse frequency (1 kHz).

To determine the mechanical property of the nanofibrous scaffolds, scaffolds were first cut into approximately 0.5-cm $\times$ 1-cm pieces and subjected to uniaxial tensile testing in the longitudinal direction by using an Instron 5544 tester (Instron, Canton, MA). Prior to testing, three thickness measurements along the length of each sample were taken with the thickness gauge and averaged; likewise, three width measurements were acquired using a digital caliper and averaged. After placing the sample in between the grips, the gauge length was measured with a digital caliper. Each sample was extended until failure at a rate of 0.1 mm/sec, and the applied force and deformation were recorded every second via Bluehill software (Instron, Canton, MA). Elastic modulus was calculated by determining the slope based on the applied load and scaffold deformation in the linear region of the stress-strain curve and the dimensions (thickness and width) of the samples.

2.3 Cell culture and in vitro cell experiments

Human mesenchymal stem cells (hMSCs) (Lonza Walkersville, Walkersville, MD) were cultured and expanded in hMSC maintenance medium (MSCGM, Cambrex, East Rutherford, NJ) and maintained in a humidified incubator at 37°C with 5% CO₂.

Scaffolds from the 7 groups of membranes ((1) control (non-ablated); (2) 50-μm diameter/50-μm spacing (50-50); (3) 50-μm diameter/200-μm spacing (50-200); (4) 100-μm diameter/50-μm spacing (100-50); (5) 100-μm diameter/200-μm spacing (100-200); (6) 200-μm diameter/50-μm spacing (200-50); and (7) 200-μm diameter/200-μm spacing (200-200)) were cut into 0.5-cm × 0.5-cm squares and secured to non-tissue-culture-treated polystyrene dishes via double-sided tape. Prior to cell seeding, these membranes were disinfected in 70% ethanol under germicidal ultraviolet light for 30 minutes, and subsequently washed with sterile deionized water for five times. The membranes were then coated with 1% fibronectin (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C and washed once with PBS before cell seeding. Approximately 3,500 cells were seeded onto each of the membranes followed by 30 minutes of incubation to allow the cells to settle and attach. After incubation, sufficient media (Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA) and 1% penicillin/streptomycin (P/S) antibiotic mixture was added to each dish containing a cell-seeded membrane. To visualize cell adhesion and morphology, samples were fixed after 24 hours with 4% paraformaldehyde (PFA), permeabilized with 0.5% Triton X-100, blocked with 3 mg/ml bovine serum albumin (BSA) + 0.1% Triton X-100, and stained with AlexaFluor 488 phalloidin for actin filaments as previously described [22]. Fluorescence images of the stained hMSCs were obtained with a Zeiss confocal fluorescence microscope (Zeiss LSM710, Carl Zeiss MicroImaging, Thornwood, NY).

To quantify cell proliferation, samples were prepared as described above. Scaffolds were seeded with a lower density of approximately 1,000 hMSCs per scaffold, followed by 30 minutes of incubation for cells to settle and attach. After incubation, sufficient DMEM supplemented with 10% FBS and 1% P/S was added to each dish containing a cell-seeded membrane. The samples were fixed after 24 hours and subsequently immunostained for the proliferation marker Ki-67 (Abcam, Cambridge, MA) along with 4',6-diamidino-2-phenylindole (DAPI) for nuclear counterstain. Fluorescence images of the stained hMSCs were obtained with the Zeiss confocal fluorescence microscope. The percentage of cells that were in the active phases of cell cycle was determined and correlated to cell proliferation rate.

2.4 Rat subcutaneous cellular infiltration model

All experimental procedures with animals were approved by the ACUC committee at University of California, Berkeley. Three membranes, approximately 0.5-cm × 0.5-cm in size, representing each of the 7 groups were implanted in the subcutaneous cavity of Sprague-Dawley rats. Briefly, all membranes were disinfected in 70% ethanol under germicidal ultraviolet light for 30 minutes, and subsequently washed five times with sterile deionized water. Prior to scaffold implantation, rats were anesthetized with isoflurane, and the incision sites were marked and disinfected with 70% ethanol. Four incisions were made on the right side and three incisions on the left side of the lower abdominal wall so that one scaffold from each of the 7 groups was implanted and placed subcutaneously relative to the incision in the same rat. Interrupted 5-0 Monocryl (Ethicon, Inc., Somerville, NJ) mattress sutures were used to close the incision.

All animals were monitored daily by a veterinarian and no adverse incidents were observed in any of the animals. At the end of the 1-week study on day 7 and the 2-week study on day 14, the rats were returned to the operating room, where they were given general anesthesia and disinfected with 70% ethanol at the initial implantation locations. The scaffolds, along with their immediate surrounding tissue, were explanted and immediately fixed with 4% PFA before being embedded in optimal cutting temperature (OCT) compound (TissueTek, Elkhart, IN) on dry ice. Cross-sections of 12- μ m thickness were generated in the transverse plane using a -20°C cryosectioner. The samples were fixed with 4% PFA, permeabilized with 0.5% Triton X-100, and blocked with 3 mg/ml BSA + 0.1% Triton X-100. They were then immunostained for pan macrophages using mouse anti-rat CD68 (Serotec, Raleigh, NC) at 1:100 dilution; for M2 macrophages using mouse anti-rat CD163 (Serotec) at 1:50 dilution; for M1 macrophages using rabbit anti-rat CCR7 (Novus Biologicals, Littleton, CO) at 1:100 dilution; and for endothelial cells using mouse anti-rat CD31 (BD Pharmingen, San Diego, CA) at 1:100 dilution. The secondary antibody used was AlexaFluor 488 donkey anti-mouse IgG (H+L) (Invitrogen Corp., Carlsbad, CA) for CD68 and CD31, AlexaFluor 546 donkey anti-mouse IgG (H+L) (Invitrogen Corp.) for CD163, and AlexaFluor 546 goat anti-rabbit IgG (H+L) (Invitrogen Corp.) for CCR7, all at 1:500 dilution. All antibodies were diluted in blocking serum. All samples were subsequently counterstained with DAPI. Fluorescent signals were observed under the Zeiss LSM710 confocal microscope. A minimum of 6 cryosections were examined for each membrane to confirm the consistency between sections.

2.5 Statistical Analysis

The data are presented as mean $\pm$ standard deviation (SD). All data were compared by using one-way ANOVA tests. Two-tailed Student's *t* test was used to test for significant differences between the groups with the same hole diameter. Holm's *t* test was then used to perform multiple comparisons against a single control to determine significant differences between each ablated scaffold group and the control. A *P* value less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Characterization of femtosecond laser-ablated nanofibrous scaffolds

To characterize the ablation of electrospun PLLA scaffolds, the effects of varying laser energy and pulse number on ablated pore size and depth, respectively, were examined (Fig. 1). Our plots show that the diameter of ablated pores increased logarithmically as laser energy increased (Fig. 1G), and the resulting depth was linearly proportional to the pulse number (Fig. 1H). With a 35 μ J FS laser, the average depths of ablated pores increased from about 21 to 130 μ m as pulse increased from 1 to 8 pulses, with complete through-holes achieved beyond 8 pulses. We demonstrated that the scaffold material could be removed by layers in controllable amounts and that well-defined pore size and spacing could be easily produced in a rapid format. Applying the optimal parameters, we prepared a total of 7 groups of membranes: (1) control (non-ablated); (2) 50- μ m diameter/50- μ m spacing (50-50); (3) 50- μ m diameter/200- μ m spacing (50-200); (4) 100- μ m diameter/50- μ m spacing (100-50); (5) 100- μ m diameter/200- μ m spacing (100-200); (6) 200- μ m diameter/50- μ m spacing (200-50); and (7) 200- μ m diameter/200- μ m spacing (200-200).

Our SEM visualization confirmed that FS laser ablation of electrospun scaffold resulted in minimal thermal stress and collateral damage (Fig. 1A-F and Fig. 2A-F). The PLLA fiber architecture, especially in regions immediately adjacent to the ablation site, did not reveal molten morphology. The ablated scaffolds maintained an overall intact fibrous structure and provided a more porous structure not only because of the patterned arrays of holes but more

importantly the relatively larger pore sizes on the post-ablation edge compared to the pores on the scaffold surface. Thus, our findings suggest that the process of FS laser ablation can impart beneficial features in addition to patterned holes to electrospun scaffolds in order to not only increase pore size and improve their bulk porosity but also provide the path for cell infiltration between layers of electrospun fibers.

To assess the effect of laser-ablated hole size and density on the mechanical properties of the ablated scaffolds, we performed uniaxial tensile testing. Representative stress-strain plots of the samples tested for each scaffold group are shown (Fig. 2G,H). The elastic moduli of the ablated scaffolds varied significantly from the control scaffold, and the decrease of the spacing between the holes had more dramatic effect on elastic modulus. Despite having the same hole density of 200- μm spacing with the two larger hole sizes, only the 50-200 group (133.8 ± 9.7 MPa) had comparable elastic modulus with that of control (147.2 ± 11.8 MPa); the 100-200 (107.4 ± 9.3 MPa) and 200-200 groups (76.8 ± 5.7 MPa) were significantly weaker as a consequence of the larger hole size. Because of the much higher density of ablated holes, the elastic moduli of the 50- μm spacing groups were decreased by approximately 2 to 2.5-fold compared to their respective 200- μm spacing counterparts (76.9 ± 7.3 MPa for 50-50; 42.9 ± 7.0 MPa for 100-50; 45.1 ± 6.9 MPa for 200-50). We believe that the presence of holes changed the stress distribution and thus decreased the strength of the scaffolds. Therefore, the range of the apparent elastic moduli of the ablated scaffolds may be tailored for specific tissue engineering applications.

3.2 Effects of laser-ablated hole size and density on cell adhesion, morphology and proliferation

Previous studies have shown that the structuring of scaffold surfaces with topological microscale features affect cellular behaviors and provide directed tissue development [23, 24]. In our study, we stained for actin and Ki-67 (a proliferation marker) to visualize the morphology and assess the proliferation of hMSCs on the ablated and control scaffolds after 1-day culture. As shown in Figure 3A-G, cell adhesion on the surface of ablated scaffolds appeared unaffected as no drastic difference in cell density was observed. However, cell morphology on the ablated scaffolds was influenced by the hole size and density. On the two 50- μm diameter groups, hMSCs exhibited similar well-spread morphology compared to the cells on the control scaffold as they easily concealed the patterned holes as if no features were present (Fig. 3B,C). As the hole size increased to 100 μm and 200 μm in diameter, the cells became more elongated with fewer cells bridging across the holes while most adhered and circumferentially aligned around the ablated holes. This difference in cell morphology was more defined for the 100-50 and 200-50 groups since the cells were more spatially confined within the constrained area as a result of the combination of larger hole size and higher hole density. In contrast, although the ablated holes altered cell morphology, our results indicated that hMSC proliferation on the surface of ablated scaffolds appeared unaffected by the varying hole sizes and densities as no considerable difference was observed (Fig. 3H). Thus, our findings showed that although hMSC adhesion and proliferation on the scaffold surface were not significantly affected, their morphology was dictated by the patterned hole features.

3.3 Effects of laser-ablated holes on cell infiltration and host response in vivo

In order to better characterize the biological response to the microscale hole features, we demonstrated through our rat subcutaneous cell infiltration model that effective cell infiltration into electrospun scaffolds *in vivo* could be achieved and significantly enhanced by utilizing a FS laser system to create desired ablations and increase scaffold porosity. Here, the scaffolds were explanted after 1 week, and cross-sections were obtained and immunostained with anti-CD68 antibody for pan macrophages as well as anti-CD163 and

anti-CCR7 antibodies for M2 and M1 phenotype of macrophages, respectively, to assess the extent of immunomodulatory response, and with DAPI to visualize the distribution of infiltrated cells throughout the thickness of the scaffolds (Figs. 4 and 5, Supplementary Fig. 1). CD68 is a pan-macrophage marker; CD163 is a surface marker representative of the M2 phenotype of macrophages, which have been characterized as the anti-inflammatory, “alternatively” activated macrophages that promote immunoregulation, tissue repair, and constructive tissue remodeling; CCR7 is a surface marker representative of the M1 phenotype of macrophages, which have been characterized as the pro-inflammatory, “classically” activated macrophages associated with signs of inflammation [25-27]. Our results showed that CD68⁺ macrophages were present in the surrounding and infiltrated cells for all 6 ablated scaffold groups, with CD163⁺ M2 macrophages contributing to a large portion of the population. (Fig. 4A-G and Fig. 5). However, no drastic differences in the presence of CCR7⁺ M1 macrophages were observed in the surrounding population of cells for all 7 scaffold groups (Supplemental Fig. 1). In addition, because of the poor cell infiltration into and the low number of cells throughout the control compared to the ablated scaffolds (as indicated by DAPI staining and Fig. 4H), very few CD68⁺ macrophages, including CCR7⁺ M1 macrophages, and no CD163⁺ M2 macrophages were found within the control scaffold. Several recent studies demonstrated that porosity of biomaterial scaffold is important in that porous materials, compared to their non-porous form, healed with minimal foreign body reaction (FBR) and fibrosis when implanted *in vivo* [28-30]. Interestingly, pores of optimal size were found to be heavily infiltrated by high ratio of M2/M1 macrophages [30]. Therefore, although the exact mechanisms in which the ablations timely modulate the macrophage phenotype remain unknown, the presence of CD68⁺ macrophages, more specifically CD163⁺ M2 macrophages, in ablated regions as well as within and surrounding our ablated scaffolds suggests the importance of microscale pores and their contribution to cell ingrowth and biological scaffold remodeling.

In addition, in Fig. 4H, cell infiltration, as determined by cell density (cells/mm²), into both scaffold and hole regions for the different scaffold groups was quantified. Because host tissue was able to grow into the ablations, we noticed significantly higher cell infiltration in the holes regions compared to the scaffold regions for every scaffold group except the 200-200 group. More importantly, cell infiltration was significantly higher in the scaffold regions of ablated scaffolds compared to the control scaffold. We speculate that the ablations facilitated overall cell infiltration as the cells were able to better migrate through the ablated holes and infiltrate between layers of electrospun fibers into the scaffold from the post-ablation edges rather than from the relatively less porous surface.

Furthermore, we observed a noticeable increase in endothelial cell ingrowth of the ablated scaffolds. In a study aimed to elucidate the effects of pore size on tissue response to porous biomaterials, Marshall et al. showed that tightly controlled pore size promoted vascular ingrowth in which the degree of intra-matrix vascularization increased with decreasing pore size [31]. However, as shown in Figure 6, our CD31⁺ staining indicated endothelial cell infiltration within the ablated holes after 1 week *in vivo*. In addition, the extent of endothelial cell ingrowth with respect to increased CD31⁺ staining within the holes of the ablated scaffolds was more prominent after 2 weeks *in vivo* (Fig. 7). This is suggestive of angiogenesis as endothelial cells were able to migrate into the holes to sprout new blood vessels. In contrast, regions without ablations, such as the spacing between adjacent holes as well as the control scaffold, showed no signs of endothelial cells within the scaffolds; instead, the distribution of CD31⁺ staining was concentrated mainly in the surrounding tissue. These findings concur with previous studies that specifically engineered pore size is pro-angiogenic and can potentially induce more vascularization [32], although additional studies need to be conducted to investigate and better understand the process.

Thus, because this approach can be not only extended to various polymers that may have different effects on inflammatory responses but also combined with other techniques such as the incorporation of sacrificial fibers in composite scaffolds, the appropriate selection of polymers or matrix proteins for fabricating electrospun scaffolds is important and needs to be customized for various tissue engineering and regenerative medicine applications.

4. Conclusions

In summary, porosity is an important issue that must be addressed when utilizing electrospun meshes as tissue-engineered nanofibrous scaffolds. Here, we demonstrate that FS laser can be used to ablate electrospun scaffolds while preserving their overall structural integrity and effectively improving cell infiltration. FS laser allows better control of laser power than nanosecond laser and avoids fiber melting and consequently the blockage of porous structure. The diameter of the holes increases logarithmically with the laser energy, and the depth of the holes increases linearly with the number of the laser pulse. In addition to their effects on cell morphology *in vitro*, electrospun PLLA scaffolds with FS laser-ablated holes of varying size and density exhibit significantly better endothelial cell ingrowth and macrophage infiltration compared to the control scaffold *in vivo*. We hope to integrate the FS laser system with tissue engineering applications to not only promote cell ingrowth and accelerate tissue remodeling but also enhance the vascularization of engineered tissues, the fabrication of 3D structures, and the control of topographical features in the cellular microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Characterization of femtosecond laser ablation of electrospun scaffolds. SEM images of PLLA nanofibrous scaffolds ablated with a single pulse of a 800 nm, 100 femtosecond laser with an energy of (A) 7 μJ, (B) 35 μJ, and (C) 70 μJ were taken to determine the resulting diameter of ablation. To characterize the ablation depth, SEM images of PLLA scaffolds ablated with (D) 1 pulse, (E) 5 pulses, and (F) 10 pulses of the same femtosecond laser with constant 35 μJ energy were taken. (G) Ablated diameter (μm) versus laser energy (μJ) for single-pulse ablation was plotted. (H) Ablated depth (μm) versus pulse number for a 35 μJ, femtosecond laser was plotted. Scale bar: 100 μm.

Figure 2.

Characterization of FS laser-ablated nanofibrous scaffolds with varying hole size and density. Six groups of ablated scaffolds were prepared: (A) 50- μ m diameter/50- μ m spacing (50-50); (B) 50- μ m diameter/200- μ m spacing (50-200); (C) 100- μ m diameter/50- μ m spacing (100-50); (D) 100- μ m diameter/200- μ m spacing (100-200); (E) 200- μ m diameter/50- μ m spacing (200-50); and (F) 200- μ m diameter/200- μ m spacing (200-200). Scale bar: 200 μ m. (G) Representative stress-strain curves of the control (non-ablated) scaffold and ablated scaffolds of 50-50 (red), 50-200 (orange), 100-50 (yellow), 100-200 (green), 200-50 (blue), and 200-200 (brown). (H) Elastic moduli were calculated from the slope of the curves and the scaffold dimensions for each group (n = 4). * indicates significant difference ($P < 0.05$) between the pairs (same diameter, different spacing) of scaffold groups. # indicates significant difference ($P < 0.05$) compared to the control group.

Figure 3.

Cell adhesion, morphology, and proliferation on nanofibrous scaffolds. hMSCs were seeded onto the ablated scaffolds of (A) 50-50; (B) 100-50; (C) 200-50; (D) 50-200; (E) 100-200; (F) 200-200; and (G) control (non-ablated) scaffold. Cells were fixed after 24h and stained with AlexaFluor 488 phalloidin for actin (green) and DAPI (blue) for cell nucleus. Scale bar: 200 μm . (H) Quantitative analysis showing the percentage of cells positive for Ki-67 on each scaffold group (n=3).

Figure 4.

Pan-macrophage recruitment and infiltration into nanofibrous scaffolds in vivo after 1 week. Scaffolds were implanted subcutaneously between the superficial fascia and the contacting muscular layer in the lower abdominal region of Sprague-Dawley rats. Immunofluorescent staining of CD68 (green) for pan macrophages and counterstain with DAPI (blue) for cell nuclei of infiltrated and surrounding cells in the cross-sections of the ablated scaffolds of (A) 50-50; (B) 50-200; (C) 100-50; (D) 100-200; (E) 200-50; (F) 200-200; and (G) control (non-ablated) scaffold. White arrows indicate the location of the holes. Edges of the scaffolds are delineated by the white dashed lines. Scale bar: 100 μ m. (H) Quantitative analysis showing general cell infiltration (in cells/mm²) into the scaffold and hole regions of the different scaffold groups (n=3). # indicates significant difference ($P<0.05$) compared to the control group. * indicates significant difference ($P<0.05$) between the pairs (same

diameter, different spacing) of scaffold groups. § indicates significant difference ($P<0.05$) between the scaffold and hole regions within each scaffold group.

Figure 5.

M2 macrophage recruitment and infiltration into nanofibrous scaffolds in vivo after 1 week. Scaffolds were implanted subcutaneously between the superficial fascia and the contacting muscular layer in the lower abdominal region of Sprague-Dawley rats. Immunofluorescent staining of CD163 (red) for M2 phenotype macrophages and counterstain with DAPI (blue) for cell nuclei of infiltrated and surrounding cells in the cross-sections of the ablated scaffolds of (A) 50-50; (B) 50-200; (C) 100-50; (D) 100-200; (E) 200-50; (F) 200-200; and (G) control (non-ablated) scaffold. White arrows indicate the location of the holes. Edges of the scaffolds are delineated by the white dashed lines. Scale bar: 100 μm .

Figure 6.

Endothelial cell infiltration into nanofibrous scaffolds in vivo after 1 week. Scaffolds were implanted subcutaneously between the superficial fascia and the contacting muscular layer in the lower abdominal region of Sprague-Dawley rats. Immunofluorescent staining of CD31 (green) for endothelial cells and counterstain with DAPI (blue) for cell nuclei of infiltrated and surrounding cells in the cross-sections of the ablating scaffolds of (A) 50-50; (B) 50-200; (C) 100-50; (D) 100-200; (E) 200-50; (F) 200-200; and (G) control (non-ablated) scaffold. White arrows indicate the location of the holes. Edges of the scaffolds are delineated by the white dashed lines. Scale bar: 100 μm.

Figure 7.

Endothelial cell infiltration into nanofibrous scaffolds in vivo after 2 weeks. Scaffolds were implanted subcutaneously between the superficial fascia and the contacting muscular layer in the lower abdominal region of Sprague-Dawley rats. Immunofluorescent staining of CD31 (green) for endothelial cells and counterstain with DAPI (blue) for cell nuclei of infiltrated and surrounding cells in the cross-sections of the ablating scaffolds of (A) 50-50; (B) 50-200; (C) 100-50; (D) 100-200; (E) 200-50; (F) 200-200; and (G) control (non-ablated) scaffold. White arrows indicate the location of the holes. Edges of the scaffolds are delineated by the white dashed lines. Scale bar: 100 μm.