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Effects of silk fibroin fiber incorporation on mechanical properties, endothelial cell colonization and vascularization of PDLLA scaffolds

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Abstract

Attainment of functional vascularization of engineered constructs is one of the fundamental challenges of tissue engineering. However, the development of an extracellular matrix in most tissues, including bone, is dependent upon the establishment of a well developed vascular supply. In this study a poly-D,L-lactic acid (PDLLA) salt-leached sponge was modified by incorporation of silk fibroin fibers to create a multicomponent scaffold, in an effort to better support endothelial cell colonization and to promote *in vivo* vascularization. Scaffolds with and without silk fibroin fibers were compared for microstructure, mechanical properties, ability to maintain cell populations *in vitro* as well as to permit vascular ingrowth into acellular constructs *in vivo*. We demonstrated that adding silk fibroin fibers to a PDLLA salt-leached sponge enhanced scaffold properties and heightened its capacity to support endothelial cells *in vitro* and to promote vascularization *in vivo*. Therefore refinement of scaffold properties by inclusion of materials with beneficial attributes may promote and shape cellular responses.

Introduction

Tissue engineering aims to restore, regenerate or replace tissues functionally compromised by injury or disease. The main strategy comprises scaffolds for tissue support combined with cells or growth factors to exploit physical cues [1–3].

Use of engineered tissues is becoming commonplace in clinics for thin tissues and for tissues characterized by limited metabolic demand (e.g. cornea [4], skin [5,6], cartilage [7–

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9)). For tissue engineering to be a viable treatment option for more complex tissues, it is imperative that they can become vascularized. In fact the lack of functional vascularization of the engineered constructs can be an indicator of clinical failure [10–16].

Bone is a highly vascularized tissue. Physiologically, osteogenesis is preceded by angiogenesis, the growth of new blood vessels from pre-existing vessels. It is therefore important to achieve rapid development of an adequate blood supply, considering the central role that the vasculature plays in tissue healing. Limited oxygen and nutrient diffusion is associated with decreased cell growth and survival. Moreover, the vasculature is involved in the production of growth factors, fundamental in the recruitment, differentiation and behavior of cells that are crucial for bone formation and bone healing [17–21].

To overcome these problems, two main strategies have been proposed to promote vascularization of tissue constructs [16,18,22]. The first is scaffold-based and takes advantage of biological [23,24] or synthetic [25–27] scaffolds. In the scaffold-based strategy, materials and structures determine biological outcomes. A promising approach is biomimicry, a scientific design discipline that emulates natural morphologies and architectures. The second strategy is cell-based and constitutes two different approaches: one is to prevascularize the scaffold [28–32] and the other is to promote angiogenesis *in vivo* [33]. The cell-based strategies include *in vitro* pre-vascularization, in which the constructs are seeded with endothelial cells and *in vivo* pre-vascularization, where constructs are implanted into a primary host to vascularize before implantation into the secondary host. Other cell based approaches exploit the addition of growth factors (e.g. vascular endothelial growth factor, fibroblast growth factor) [34,35] or peptides derived from adhesion proteins or extracellular matrix molecules (e.g. laminin, fibronectin, osteopontin) [36,37] or co-culture systems [38–40]. The challenge is always to promote tissue healing and regeneration, while preventing ischemia and nutrient loss.

The aim of this work was to refine a bone tissue engineering baseline scaffold, enhancing its capacity to support endothelial cells and to promote vascularization. In recent years biomimesis, the mimicking of the architecture of the extracellular matrix (ECM), has been a major approach explored for 3D scaffold development. The ECM is a fibrous composite of nano and micro fibrils which surrounds cells and comprises many biological components. The biomimetic matrix should ideally provide cell adhesion sites to maintain cell phenotype and function, mechanical strength, a biodegradation rate compatible with tissue generation and a controlled degree of inflammation [3,28,41–43]. In particular, biomaterial implants should influence the host inflammatory and reparative response [44].

The idea to combine different structures and materials to produce multicomponent scaffolds to improve the control of mechanical properties and metabolic cell activity has been recently exploited. To satisfy these demands, among all the strategies, multi-layered composite scaffolds, embed textile materials within hydrogels or bulk polymers were proposed [45,46].

In our study a multicomponent scaffold was developed, combining the properties of a synthetic porous scaffold with the properties of a natural fibrous structure to enhance bioactivity. Silk fibroin fibers were incorporated into salt-leached sponge without modifying scaffold porosity. The baseline scaffold, composed of poly-D,L-lactic acid (PDLLA), was specifically designed to promote ECM production and mineralization providing a perfect 3D porous structure as support [47,48]. Silk fibroin fibers were selected for their ability to support endothelial cell attachment and growth. Unger et al. demonstrated the capability of silk fibroin fibers to favor the formation of microcapillary-like structures *in vitro*. The authors also reported a rapid anastomosis of preformed capillaries with the host vasculature and a rapid vasculature growth into the silk construct thus leading to the vascularization *in*

vivo [39,40,49]. Moreover silk fibroin fibers have been characterized by a gradual degradation attributable to macrophages and multinucleated giant cells, which in turn can produce pro-angiogenic molecules [42,50]. Our hypothesis was that the capacity of PDLA sponge to support endothelial cells *in vitro* and to promote vascularization *in vivo* could be improved by addition of silk fibroin fibers. In addition, the properties of both scaffolds were investigated.

Materials and Methods

Scaffold production

Two experimental groups were established; Group 1 (control scaffold) and Group 2 (scaffold with fibers).

Group 1 was produced by the solvent casting particulate leaching technique [51]. PDLA (Resomer R207S, Boehringer Ingelheim, Germany) was dissolved in dichloromethane:dimethylformamide (70:30 v/v) and a 7% (w/v) solution was prepared. Sieved NaCl particulates, ranging from 425 to 1180 μm , were mixed with PDLA, obtaining a 1:25 w/w polymer/porogen final concentration. The mixture was air dried (24–36 hours) and immersed in deionized water (dH_2O) for 3 days with changes every 6 hours for the removal of the porogen.

In parallel, fibers were introduced into the scaffold (Group 2). The fibroin nets were produced from *Bombyx mori* silkworm cocoons. Briefly, cocoons were degummed by boiling twice, 1 hour each, in an aqueous solution of Na_2CO_3 (1.1 and 0.4 g/l, respectively), in order to remove sericin. Degummed silk fibers were then washed several times in distilled water and dried at room temperature. Then 0.05 g of these spread fibers was mixed with sieved NaCl particulates, ranging from 425 to 1180 μm as before, and the PDLA solution (as above), obtaining a 1:14 w/w polymer/porogen final concentration. The mixture was air dried (24–36 hours) and immersed in deionized water (dH_2O) for 3 days with changes every 6 hours for the removal of the porogen. Before use, scaffolds were cut into discs of 8 mm diameter, sterilized with aqueous ethanol solution 70% (v/v) and dried under a sterile hood at room temperature.

Scaffold characterization

Scanning electron microscopy (Supra 40, operating mode: high vacuum, secondary electron detector, Carl Zeiss, Europe) was used for the evaluation of scaffold morphologies. Samples were previously sputter-coated with a thin gold layer under argon (SEM Coating Unit PS3, Assing S.p.A., Rome, Italy).

A micro-CT system (μCT 40, Scanco Medical, Bassersdorf, Switzerland) was used to image and quantify the 3D microstructure of each sample as previously described [52]. Briefly, the samples ($n=4/\text{group}$) were scanned at 12 μm voxel resolution with an integration time of 250 ms. Isotropic slice data were obtained and reconstructed into 2D images. These slice images were compiled and analyzed to render 3D images and obtain quantitative architectural parameters. Regions of interest were selected in each slice image and thresholded to eliminate background noise. A threshold to distinguish polymer material from pore space and background noise was chosen and kept consistent throughout all evaluations. Porosity, pore size distribution and degree of anisotropy were evaluated. After measuring the cross-sectional area, wet scaffolds ($n=4/\text{group}$) soaked in PBS for 30 minutes were mechanically tested. Compressive mechanical testing was performed using a uniaxial electromechanical testing device (Info 650R, DDL, Eden Prairie, MN). Initial scaffold height was measured using a micrometer. Samples were tested to failure at a rate of 1 mm/minute. Standard solid compression plates were used for testing. A preload of approximately 1N was applied to the

samples before initiating compression testing. Stress vs strain data were computed from load and displacement measurements, and compressive modulus was determined based on the slope of the entire linear-elastic region of the stress–strain curve.

***In vitro* cell culture**

After sterilization, scaffolds were placed in 48 well plates and washed with PBS. Each sample was seeded with 20 µl media containing 75,000 Human Umbilical Vein Endothelial Cells (HUVEC CC-2517, Lonza, Walkersville, MD) expanded to passage 4. After 24 hours, scaffolds were moved to 24 well plates and incubated in EGM-2 media (Lonza). All scaffolds were cultured in complete endothelial growth media (EGM-2 media) with FBS, bFGF, VEGF, insulin-like growth factor (IGF), ascorbic acid, epidermal growth factor (EGF), gentamycin sulphate and amphotericin-B (GA- 1000) and heparin (all from Lonza SingleQuots) at 37°C, 5% CO₂. Media were replaced every 2 days for a total of 21 days. Scaffolds were harvested at different experimental time points.

Proliferation

Cell number variations over time in culture were determined using a Picogreen DNA quantification assay (Quant-iT PicoGreen dsDNA Assay Kit, Invitrogen, Grand Island, NY). At pre-determined time points, medium was removed from wells and scaffolds washed three times with PBS. 500 µl of 0.05% Triton-X/PBS was used to completely submerge the scaffold before freezing and storing at –20°C until analysis. Each thawed sample was then sonicated for 10 s using a Virsonic ultrasonic cell disrupter (Virtis, Warminster, PA). Samples (100 µl, in triplicate) were aliquoted into a black 96 well plate and 100 µl PicoGreen working solution added to each well. The plate was read on a Perkin Elmer HTS 7000 fluorescent plate reader (excitation wavelength: 485 nm, emission wavelength: 535 nm). To calculate DNA concentrations, a calibration curve was generated using the double stranded DNA standard provided with the kit. Results were divided by 7.7 pg DNA, (the average amount of DNA in a cell [53]), to obtain estimates of cell number.

Cell viability

The cellular metabolic activity was measured using the CellTiter Blue assay (Promega, Madison, WI) which is based on the ability of living cells to reduce the non-fluorescent compound resazurin to the fluorescent compound resorufin. The assay was performed according to the manufacturer's instructions. In brief, the seeded scaffolds were incubated for 3 hours at 37°C with phenol red free culture medium supplemented with Titer Blue diluted 1:5. The resulting fluorescence was read on a fluorescence reader (excitation wavelength: 550 nm; emission wavelength: 595 nm).

Immunofluorescent localization of PECAM-1 expression on endothelial cells

Cells were fixed with formaldehyde (4% w/v in PBS) for 15 min at room temperature, washed with PBS and incubated in blocking buffer (6% BSA / 10% donkey serum / PBS) for a hour. Subsequently the samples were incubated with 1° antibody against Platelet Endothelial Cell Adhesion Molecule (1:100, PECAM-1, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. After washes in PBS, the samples were incubated in 2° antibody (1:1000, donkey anti-rabbit Alexa Fluor 568, Molecular Probes, Grand Island, NY) for 1.5 h at room temperature. The scaffolds were washed again in PBS and permeabilized with 0.25% Triton-X 100 in PBS for 20 min. This was followed by a brief rinse in PBS and a nuclear counterstain (DAPI, Sigma Aldrich, St Louis, MO), according to the manufacturer's protocol. After the final rinses with PBS, samples were examined by confocal microscopy (Zeiss LSM 510 UV).

RNA isolation and reverse transcriptase–quantitative PCR

VWF (Von Willebrand Factor) and PECAM-1 genes were assessed by RT-qPCR using a Custom PCR Array (RT² Profiler PCR Array CAPH10845; SABiosciences, Qiagen, Valencia, CA). Total RNA from scaffolds (3 replicates for each group) was extracted using the RNeasy MINI kit and QIAshredder columns (Qiagen) according to the manufacturer's instructions. The total RNA was quantified using spectrophotometry (Nanodrop ND-1000, Thermo Scientific, Wilmington, DE) and ranged from 1.2 to 6 µg. For best results on the PCR array, all RNA samples were selected for purity using a A260/A280 UV spectrophotometry ratio greater than 1.99. 250 ng RNA was used for cDNA synthesis, performed using the RT² First Strand Kit (SABiosciences, Qiagen) according to the manufacturer's protocol. The cDNA was then amplified using RT² qPCR Mastermix with RT² Profiler PCR Arrays (SABiosciences, Qiagen). The results were normalized to 3 housekeeping genes (HPRT1, GAPDH and RPL13A). Relative gene expression values were analyzed using the RT² Profiler PCR Array Data Analysis version 3.5 web-based software package.

Subcutaneous implants, Microfil perfusion, and micro-CT images

All animal procedures were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee (IACUC # A10021). Female 9–10 week-old Sprague Dawley rats (Charles River, Wilmington, MA) were housed two per cage and had access to chow and water ad libitum. All scaffolds used for implantation were prepared in a sterile biosafety cabinet and equilibrated in alpha MEM medium (no additives) for 12 h. Each of 20 rats received four randomized dorsal subcutaneous implants. This was performed by making two lateral incisions on the dorsal region and using a custom made tunneling tool to position the implant at a distance from the incision site. After surgery the animals were given a single dose of 0.03 mg/kg buprenorphine for pain relief. In all instances, the implantation sites healed without complications.

The vascular network in the scaffold area was investigated at 3 and 6 weeks post surgery using a modified version of a previously described micro CT-based angiography technique [54]. After induction of anesthesia using 5% isoflurane, rats were maintained at 2% isoflurane and the thoracic cavity was opened to insert an 18 gauge catheter (SURFLO® Teflon IV catheter, Terumo Medical) through the left heart ventricle into the ascending aorta. The inferior vena cava was cut and 0.9% saline was perfused through the vasculature using a peristaltic pump (Masterflex, Cole-Parmer, Vernon Hills, IL) until complete clearance was achieved. Then 0.9% saline containing 0.4% (w/v) papaverin hydrochloride was perfused. The rats were then perfused with 10% neutral buffered formalin (NBF) for 5 minutes to fix the vasculature. Then polymerizable, lead chromate based, radiopaque contrast agent (Microfil MV-122, Flow Tech, Carver, MA) was syringe-injected. The contrast agent was prepared according to manufacturer instructions but diluted to 66 % (vol/vol) with diluent and curing agent. Samples were stored at 4°C overnight to allow polymerization of the contrast agent before implant retrieval. Explants were rinsed in PBS, immersion fixed in 10% neutral buffered formalin for 48h prior to imaging in the Viva-CT system (Scanco Medical).

As previously described, isotropic slice data were obtained and reconstructed into 2D images. These slice images were compiled and analyzed to render 3D images and obtain quantitative architectural parameters. Regions of interest were globally thresholded based on X-ray attenuation and 3D images of the vascular network segmented from the surrounding tissues. Harvested scaffolds were evaluated to quantify the vessel volume, connectivity, number, thickness, and degree of anisotropy.

Histological analysis. Rats that were not perfusion fixed were euthanized by CO₂ asphyxiation. Explants (from perfused rats and fresh tissue) were immersion fixed in 10% NBF. The tissues were processed and paraffin embedded for histological analysis. Sections, cut at 5 μ m, were deparaffinized and rehydrated to stain with hematoxylin and eosin (H&E) and imaged with brightfield microscopy to assess cellular organization. Representative sections were stained with 1% picosirius red and observed under polarized light to identify collagen fiber organization and maturation.

Statistical analysis

The data are expressed as mean $\pm$ standard deviation (mean $\pm$ SD). Statistical analyses were performed using Prism 5 (GraphPad, San Diego, CA). To determine the statistical significance of the results one-way and two-way ANOVA tests were performed (if the interaction term was significant). F-tests of the equality of the variances were conducted with a significance level of 0.05. Significance was assigned at *p* values less than 0.05. For RT-qPCR analysis, the RT² Profiler PCR Array Data Analysis version 3.5 web-based software package was used.

Results

Scaffold morphology and mechanical properties

The two scaffold typologies were produced by the solvent casting particulate leaching technique with or without silk fibroin fibers. NaCl concentration was adjusted between the two groups to achieve the same porosity. Ultimately a concentration of 1:25 w/w polymer/porogen in group 1 (PDLLA) and 1:14 w/w polymer/porogen in group 2 (PDLLA + silk fibroin fibers) was considered optimal. Resulting structures are shown in Figure 1A. The analysis of the SEM images revealed that the fibers were randomly distributed and well dispersed within the PDLLA sponge.

Micro-CT analysis generated slice information on the scaffolds and color graded images of the pore size distribution. Reconstructed micro-CT 3D images provided a quantitative morphometric analysis of the scaffolds. Accordingly, the porosity and the pore size distribution were determined and are presented in Figures 1B and C. The porosities were comparable in the two groups and in excess of 80%. Interestingly, the pore size distribution was also similar and no big differences were observed in the peaks or in the curve profiles of the two groups. Finally, scaffolds with silk fibroin fibers (group 2) had significantly higher degree of anisotropy (Figure 1D) compared to group 1, indicating that group 2 is characterized by a preferential material direction.

Stresses and strains were plotted from the compression data. Compressive moduli were calculated from the slope of the linear fit to the elastic range of the stress-strain curve and the results (Figure 2) demonstrate that the scaffolds with fibers have more than double the elastic modulus of the control scaffolds.

HUVECs proliferation, metabolic activity, distribution and gene expression

7×10^4 HUVECs were seeded onto each scaffold and cultured in conditions promoting angiogenesis. Retention and growth of endothelial cells were considered important indications of scaffold properties.

The retention of cells on the two different scaffold groups was comparable (Figure 3A, day 1). At day 7 there was a decrease in cell number which was probably due to a physiological adaptation to the new environment and group 2 scaffolds were characterized by a lower

amount of cells. However, it appears that proliferation was favored in the scaffold with silk fibroin fibers and at 21 days the number of cells was significantly higher in that group.

Cell viability was determined during the cell culture period using the CellTiter-Blue assay. The metabolic activity (viability normalized to cell number) was comparable in the cells seeded on the two different scaffolds at each time point (Figure 3B). The profile was similar in the two groups: an increase in metabolic activity was observed after day 1 with the peak at day 7.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) analyses (Figure 4) showed that the gene expression of the cells seeded onto the two different scaffolds at 7 and 21 days was comparable, based on VWF and PECAM-1 mRNA levels. No significant differences in gene expression were observed between the two scaffold groups at either the 7 or 21 day timepoints. Confocal microscopy and immunolocalization were used to evaluate cell distribution and PECAM-1 protein expression at cell-cell junctions. Cells attached to both scaffolds. In group 1, cells did not express a strong PECAM-1 signal, and after 21 days it was only present around pore edges. In group 2, cells seemed to prefer to attach to the fibers, then, as their number increased, they colonized not only the fibers but the spaces in between. PECAM-1 expression appeared qualitatively higher with increasing days in culture in scaffolds with silk fibroin fibers.

Subcutaneous model, histology and perfusion

Rats were perfused with a vascular contrast agent to quantify blood vessel ingrowth 3 or 6 weeks after acellular construct implantation. Examination of the explanted samples revealed that scaffolds became vascularized and that scaffold materials were not completely degraded.

The subcutaneous implants were stained with hematoxylin and eosin (Figure 6A) to show nuclei and possible cellular infiltration. Histological analyses showed tissue ingrowth into both the scaffolds. Moreover multinucleated giant cells (macrophages) were visible at the interface between new tissue and the material especially in group 2 at 3 weeks. A comparison of group 1 with group 2 after 3 weeks of culture shows that qualitatively more blood vessels are present in the scaffold with silk fibroin fibers, as evidenced by black spots of perfused contrast agent.

The organization of newly formed collagen matrix was evaluated using picrosirius red staining and polarized light microscopy. In both groups a fibrous capsule formed around the implanted scaffold. The capsule did not block tissue ingrowth into the scaffolds and collagen (birefringent yellow/orange fibers) was partially oriented and aligned in each group.

Perfused scaffolds were carefully harvested, cleaning them from the surrounded tissue to restrict evaluation to the vessels inside the scaffold. Figure 7A shows the vessels (dark gray) which have invaded the scaffold area (light gray). Micro-CT analysis revealed a significantly greater vessel volume normalized to scaffold volume in group 2 compared to group 1 at 3 weeks (Figure 7B). At 6 weeks, the vascular volume was comparable in the two groups. In Figure 7C representative scans of perfused constructs of both groups at 3 and 6 weeks after implantation are shown. The diameters of the vessels are displayed on a color scale. As before, there is a greater vascular volume in group 2 at 3 weeks, whereas there is no difference between the groups at 6 weeks.

Finally other morphological parameters such as connectivity, vessel number, and degree of anisotropy were evaluated. The measure of the interconnectedness of the vascular structures (Figure 8A) and the number of distinct vessels (Figure 8B) were not significantly altered by

the presence of silk fibroin fibers. However the addition of silk fibroin fibers favored a higher degree of anisotropy, indicating a preferential orientation of the vessels (Figure 8C).

Discussion

Rapid and sufficient integration of implants with the host vasculature, to ensure an adequate supply of oxygen and nutrients, remains a significant challenge in tissue engineering. This study demonstrates that modification of a baseline scaffold resulted in improved mechanical properties and accelerated tissue integration and vascularization *in vivo*.

First we demonstrate the ability to generate two different constructs (PDLLA alone or PDLLA with silk fibroin fibers), such that stiffness was increased upon addition of fibers but porosity remained the same. The compression testing results show that the scaffolds with fibers are two-fold stiffer than the PDLLA alone. The increase in apparent elastic modulus achieved in scaffolds with the fibroin fibers could be attributed to their structure, in which triaxial stresses that are established during compression, are also relatively high in tension. Therefore, the addition of an oriented structure, such as the fibroin fibers, could provide benefits in terms of mechanical properties even if positioned randomly or longitudinally to the applied load, as demonstrated in a reticular structure [55].

In culture, we showed that cell proliferation at 21 days was higher for group 2, probably due to the active domains of silk fibroin. In fact, fibroin protein peptides have been demonstrated to enhance cell growth [56]. We also showed that, even if gene expression was unaffected, the adhesion molecule PECAM-1, a well-known endothelial marker, was much more extensively located on the membrane of cells seeded onto the scaffolds with silk fibroin fibers. One explanation may have been related to the amino acid sequences of the fibroin molecule that have been shown to support endothelial cell phenotype and activity [28,57]. Furthermore, it has to be considered that the gene expression results were normalized to housekeeping genes and, for this reason, the higher cell number of HUVECs supported by the group 2 scaffolds at day 21 would partially explain the more PECAM-1 observed as the cells are more crowded. The immunolocalization of PECAM-1 mechanoreceptor to the cell-cell junction at 21 days suggests a higher degree of organization of the endothelial cells in group 2, proving that scaffold with silk fibroin fibers better support endothelial cells in static culture.

The vascularization of the scaffolds *in vivo* is of great importance for understanding the possible applications of this material. Recent studies have underscored how differences in the inflammatory response can lead to differences in tissue repair. Macrophages and multinucleated giant cells play a major role in scaffold vascularization by virtue of their secretion of pro-angiogenic factors [18,40,58]. In our study, it is evident that the implantation of both scaffolds caused an inflammatory response, highlighted by the fibrotic capsule encircling all the scaffolds and by the presence of giant multinucleated cells (even more prominent in the scaffolds with silk fibroin fibers). It is a fair assumption that the silk fibroin fibers interacted with macrophages and multinucleated giant cells in order to accelerate the vascularization process at 3 weeks. However, the *in vivo* perfusion results show that a fully integrated host vasculature formed in the acellular scaffolds of both groups, indicating an earlier and faster integration of scaffolds from group 2.

The other significant parameter different in the two groups is related to vessel anisotropy. In the scaffold with silk fibroin fibers, vessels had a preferred direction in agreement with the scaffold anisotropy. It seems that, even if the fibers had been randomly distributed, the general structure in group 2 shows some level of organization, guiding the orientation of the vessel formation.

Conclusions

This study demonstrated that adding silk fibroin fibers to a PDLLA salt-leached sponge increased scaffold stiffness, and heightened its capacity to support endothelial cells *in vitro* and to promote vascularization *in vivo*. The immunolocalization of PECAM-1 mechanoreceptor to the cell-cell junction at 21 days suggests that scaffold with silk fibroin fibers better support endothelial cells in static culture. Additionally the *in vivo* perfusion results indicate that a fully integrated host vasculature formed in the acellular scaffolds of both groups, with faster vascularization of the composite scaffolds. Further studies using segmental bone defect models and considering both vascularization and mineralization are warranted to better characterize the role of silk fibroin fibers added to a PDLLA scaffold in the bone healing process.

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Figure 1.

A) From left to right: Scanning electron microscopy images, cross-sectional micro-CT slices and color-coded pore diameter scale images of the two scaffolds. B) Porosity of the two scaffolds. C) Pore size profile of the two scaffolds. D) Degree of anisotropy of the two scaffolds. Group 1: PDLLA salt-leached sponge; Group 2: PDLLA salt-leached sponge with silk fibroin fibers.

Figure 2.

A) Stress-strain plot of a representative compressive mechanical testing for a PDLLA salt-leached sponge scaffold with linear fit slope shown. B) Elastic moduli of wet scaffolds after immersion in PBS for 30 min. *, $p < 0.05$. B) Representative stress-strain plot for compressive mechanical testing at a rate of 1mm per minute for a sponge scaffold. Group 1: PDLLA salt-leached sponge; Group 2: PDLLA salt-leached sponge with silk fibroin fibers.

Figure 3.
 (A) HUVEC cell number. *, $p < 0.05$. (B) Viability index for HUVEC cell culture (normalized to cell number). \$, $p < 0.05$ vs the same scaffold at the previous time point. Group 1: PDLLA salt-leached sponge; Group 2: PDLLA salt-leached sponge with silk fibroin fibers.

Figure 4.

RT-qPCR analysis of VWF and PECAM-1 gene expression in cells seeded onto the scaffolds at different time points: A, 7 days; B, 21 days. No significant differences are present (n=3). Group 1: PDLLA salt-leached sponge; Group 2: PDLLA salt-leached sponge with silk fibroin fibers.

Figure 5. Confocal Laser Scanning Microscopy images of HUVEC cells stained for nuclei (DAPI, blue) and PECAM-1 (red). Group 1 and Group 2 at 7, 14 and 21 days after cell seeding are shown. Silk fibroin fibers stain with DAPI. Scale bars = 100 μ m. Group 1: PDLLA salt-leached sponge; Group 2: PDLLA salt-leached sponge with silk fibroin fibers.

Figure 6.

Histological images of sections (5 μm) of explanted scaffolds. Left pictures (A) show samples stained with hematoxylin and eosin at 3 and 6 weeks with sample at 3 weeks perfused with vascular contrast agent. Black arrows indicate multinucleated giant cells. Right pictures (B) show samples stained with picrosirius red at 3 weeks. Center area indicates a inner part of the implants, edge area the interface between the implants and newly formed tissue. Scale bars = 100 μm . Vascular contrast agent is also seen in the center area images in B. Group 1: PDLA salt-leached sponge; Group 2: PDLA salt-leached sponge with silk fibroin fibers.

Figure 7.

(A) Micro-CT images of scaffold implanted subcutaneously in rat perfused with Microfil vascular contrast agent. Vascature (dark grey) growing into implant area (light grey), (B) Quantification of vascular volume/total scaffold volume. *, $p < 0.05$ (C) Representative scans of blood vessels 3 and 6 weeks after implantation. Color scale bars, defining vessel diameters, range from 0 to 320 μ m at 3 weeks to 0 to 240 μ m at 6 weeks. Group 1: PDLLA salt-leached sponge; Group 2: PDLLA salt-leached sponge with silk fibroin fibers.

Figure 8.

(A) Vascular connectivity, (B) Number of vessels and (C) Degree of anisotropy of scaffolds at 3 and 6 weeks after implantation. *, $p < 0.05$. Group 1: PDLLA salt-leached sponge; Group 2: PDLLA salt-leached sponge with silk fibroin fibers.