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Nanosecond laser ablation enhances cellular infiltration in a hybrid tissue scaffold

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ABSTRACT

Hybrid tissue engineered (HTE) scaffolds constituting polymeric nanofibers and biological tissues have attractive bio-mechanical properties. However, they suffer from small pore size due to dense overlapping nanofibers resulting in poor cellular infiltration. In this study, using nanosecond (ns) laser, we fabricated micro-scale features on Polycaprolactone (PCL)-Chitosan (CH) nanofiber layered bovine pericardium based Bio-Hybrid scaffold to achieve enhanced cellular adhesion and infiltration. The laser energy parameters such as fluence of 25 J/cm^2, 0.1 mm instep and 15 mark time were optimized to get structured microchannels on the Bio-Hybrid scaffolds. Laser irradiation time of 40 μs along with these parameters resulted in microchannel width of ~50 μm and spacing of ~35 μm between adjacent lines. The biochemical, thermal, hydrophilic and uniaxial mechanical properties of the Bio-Hybrid scaffolds remained comparable after laser ablation reflecting extracellular matrix (ECM) stability. Human umbilical cord mesenchymal stem cells and mouse cardiac fibroblasts seeded on these laser-ablated Bio-Hybrid scaffolds exhibited biocompatibility and increased cellular adhesion in microchannels when compared to non-ablated Bio-Hybrid scaffolds. These findings suggest the feasibility to selectively ablate polymer layer in the HTE scaffolds without affecting their bio-mechanical properties and also describe a new approach to enhance cellular infiltration in the HTE scaffolds.

1. Introduction

Tissue engineering (TE) is a widely used approach to fabricate biomimetic scaffolds resembling natural ECM intended for regeneration of various types of tissues [1]. In the past, fabrication of various tissue-engineered constructs has been reported using polymers and decellularized tissues [2–6]. Hybrid tissue engineered scaffolds that combine the properties of natural three-dimensional (3-D) tissues and biocompatible synthetic polymers are recently gaining popularity as they bring together the advantages of both constituents [7–10]. Natural 3-D topologies of tissues support cell adhesion and proliferation, which is crucial to maintain balance between the rate of degradation of scaffold and neoextracellular matrix formation [11–13]. Whereas, synthetic polymers, which are an active component of the HTE scaffolds provide attractive tunable mechanical properties that are in turn deposited on biological tissues by either electrospinning or dip-coating [11–13]. Electrospinning is preferred for HTE over other techniques like dip coating to deposit polymeric nanofibers on biological tissues since it provides desired fiber diameter and solvent evaporation without causing any impairment to the tissues [14–15]. These electrospun nanofibers are oriented either in random or aligned manner on the hybrid scaffolds to mimic natural ECM and enhance their mechanical properties [16]. The nanotopography of these electrospun scaffolds shows better adhesion and spreading due to which they are considered to be better 3-D systems for TE [17–23]. However, one main disadvantage with the electrospun hybrid scaffolds is their small pore size due to overlapping nanofibers layered on the dense natural tissue [10,24]. The small pore size of the electrospun hybrid scaffolds hinders cell infiltration and thus reduces the diffusion of large molecular nutrients, vascular infiltration and remodelling capacity upon implantation *in vivo* [25–26].

Various techniques like laser patterning, incorporation of sacrificial fibers, addition of porogens, modification of fiber diameter and post-processing photo-patterning or ultraviolet radiation treatment have been reported earlier to increase pore size, overall porosity, and cellular infiltration of TE scaffolds [27–28]. Some porogens also improved cellular infiltration but later caused membrane delamination and pore collapse [29]. Additionally, the photo-patterning method requires multiple step procedure and dedicated chemistry to engineer

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photosensitive materials [30]. In order to overcome these disadvantages, laser micropatterning is widely used to design patterned TE scaffolds since it is a fast non-contact technique and requires no additional chemical processing [31–32]. However, a major issue that remains with the lasers is the potential for thermal damage [31–32]. With the advances in laser technology, the use of ultrashort lasers used to ablate electrospun fibrous scaffolds, produces high irradiance pulses that result in sharp ablation, rapid vapour formation, minimal heat conduction and least possible physical stress [33]. Most of the studies that reported laser induced modifications for TE applications were focussed primarily on surface ablations such as grooves and microwells on polymer based TE scaffolds, which resulted in enhanced porosity and cellular infiltration after laser micropatterning [24,32,34–36]. Cellular infiltration in these laser-ablated scaffolds is known to promote cell growth, accelerate remodelling and vascularization, thus controlling the cellular microenvironment [24,37–40]. In addition to TE, laser technique has also been used for the ablation of soft biological tissues, which suggests feasibility to micropattern tissue based HTE scaffolds [41–43]. Such an approach has not been reported before for hybrid scaffolds.

Based on these studies, we hypothesized that it would be possible to increase cellular colonization of the HTE scaffolds by modifying them with laser ablated microchannels while retaining their bio-mechanical properties. In this study, we describe the utilization of nanosecond laser to selectively ablate a part of polymer layer in our previously described Bio-Hybrid scaffold to fabricate microchannels [10,44]. The design of the Bio-Hybrid scaffold involved deposition of an adhesive layer of PCL-CH nanofibers on the fibrous side of the decellularized bovine pericardium [10]. The Bio-Hybrid scaffold that combined bovine pericardium and aligned synthetic polymeric nanofibers exhibited favourable bio-mechanical properties, enhanced cellular adhesion and dense ECM deposition that may impart remodelling ability [44]. Hence, the optimal ablation parameters were determined for patterning microchannels on the Bio-Hybrid scaffold to increase the regenerative capacity by improved cellular ingrowth. Hybrid scaffolds were finally characterized post-ablation for surface, mechanical and biological properties *in vitro*.

2. Materials and methods

This study was approved by the Institutional Review Board and the principles outlined in the Declaration of Helsinki were followed for all human or animal experimental investigations performed. All the procedures were approved by the Institutional Animal Ethics Committee (IIT Madras, India) and the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India, were performed in accordance with the Rule 5(a) of the Breeding and Experiments on Animals (Control and Supervision; 1998). In addition, for investigations involving human subjects, informed consent was obtained from the participants involved. The study involved human umbilical cord tissue cells and was performed in accordance with the institutional guidelines approved by the Institute Ethics Committees at Indian Institute of Technology Madras, Chennai (IEC/IITM/003/2013).

2.1. Fabrication of Bio-Hybrid scaffold

We fabricated Bio-Hybrid scaffold combining decellularized bovine pericardium and Polycaprolactone (PCL)-Chitosan (CH) blend as described in our previous report with minor modifications [10,44]. Electrospinning was used to coat PCL-CH nanofibers along the axial fibers of the bovine pericardium (BP) to fabricate aligned nanofibrous Bio-Hybrid scaffolds. Briefly, BP was decellularized using 1% deoxycholic acid (D2510-100G; Sigma-Aldrich, MO, USA) for 24 h with continuous stirring at 37 °C in $PBS^{-/-}$ followed by DNase (D4527; Sigma-Aldrich, MO, USA) and RNase (R6513; Sigma-Aldrich, MO, USA). The decellularized tissue was analysed for acellularity using histology as described previously using routine hematoxylin and eosin (H&E) staining, nuclear specific 4′,6-diamidino-2-phenylindole (DAPI) staining and DNA estimation [10]. A blend of 10% Polycaprolactone (440744, Sigma-Aldrich, USA) and 1% Chitosan (417963, Sigma-Aldrich, USA) dissolved in Trifluoroacetic acid (TFA; 2029282, SISCO Research Laboratories Pvt. Ltd. India) and Dichloromethane (DCM; 0422123, SISCO Research Laboratories Pvt. Ltd. India) in the ratio of 80:20 was electrospun using a customized electrospinning device (Holmarc Opto-Mechatronics Pvt. Ltd., India; Model: HO-NFES-040). The polymer was electrospun for 2 h on the decellularized BP with a voltage of 15 kV, 0.5 ml/h and at a distance of 20 cm. The resulting polymer coated Bio-Hybrid scaffold was stabilized in 0.5 M NaOH and was finally washed and preserved in 70% ethanol for further use.

2.2. Nanosecond laser ablation of Bio-Hybrid scaffolds

An Nd^{3+} doped fiber laser system (SPI Lasers UK Ltd., 20W G3.0 Pulsed Fiber Laser) with fundamental output wavelength of 1064 nm, 25 kHz repetition rate and pulse width of 25 ns was used to ablate the Bio-Hybrid scaffolds. The 1064 nm laser output from the fiber laser was collimated and fed into a galvo scanner (SUNNY, Model no. TS8618D). The galvo scanner and the laser firing were controlled by a personal computer using the software SN Mark, version 2.0. The output laser beam from the galvo scanner was focussed on the Bio-Hybrid scaffolds using f-theta lens with a focal length of 218 mm. The laser spot size on the scaffold was calculated and found to be 54.93 μm considering M^2 value as 1.86. Three parameters reflecting the laser characteristics were varied to get the desired patterning on the scaffolds; i) laser energy fluence, ii) instep (distance between the two patterned microchannels) and iii) marking time. A laser scanning velocity of 250 mm/s, marking time of 15, 0.1 mm instep and laser energy fluence of 25 J/cm^2 were used to ablate the Bio-Hybrid scaffolds [45]. These parameters were optimized by varying laser energy levels (10 W, 12 W, 16 W and 20 W), instep (0.01 mm to 0.1 mm) and marking time (5 to 15 passes). The procedure was repeated until consistent results were obtained with a sample size of n = 3. (Detailed calculations are provided in Supplementary file [45].)

2.3. Characterization of micropatterned Bio-Hybrid scaffolds

2.3.1. Scanning Electron Microscopy

Both decellularized BP and polymer coated Bio-Hybrid ablated scaffolds were imaged (n = 3) for physical changes after laser ablation using High Resolution SEM (FEI, Hillsboro, OR, USA; Quanta 400 FEG). The acquired images were later used to compare changes in microchannel width and depth, respectively. The samples were air dried and sputter coated in vacuum with an electrically conductive 5 nm thick layer of Gold/Palladium alloy using a Precision Etching Coating system (Gatan, PA, USA; Model 682) before imaging. The width and depth of the patterned microchannels on the scaffolds were analysed from SEM images using Image J software (ImageJ: open source image processing program; imagej.nih.gov/ij). SEM was also used to image cellular attachment on the scaffolds.

2.3.2. Functional characterization

The surface chemistry of polymer coated Bio-Hybrid scaffolds before and after ablation was assessed for changes due to ablation using Attenuated Total Reflectance spectra (ATR-FTIR; Perkin-Elmer Spectrum One, USA). Spectra were taken in the wavelength region 400 cm^{-1} to 4000 cm^{-1}.

2.3.3. Differential Scanning Calorimetry

Thermal stability of the Bio-Hybrid scaffolds (n = 3) before and after ablation (4 × 4 mm^2) was demonstrated by Differential Scanning Calorimetry (DSC) in a NETZSCH DSC 204 using Pan Ag crucibles with about 3.380 mg of samples, under dynamic nitrogen atmosphere (50 and