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Behavior of valvular interstitial cells on trilayered nanofibrous substrate mimicking morphologies of heart valve leaflet

Dr. Soumen Jana* and Dr. Amir Lerman

Division of Cardiovascular Diseases Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA

Abstract

Heart valve tissue engineering could be an alternative to the current bioprosthetic heart valves and their limitations, especially for pediatric patients. However, heart valve tissue engineering remains challenging because leaflets — the primary component of a heart valve — have three diversely oriented layers — circumferential, random and radial. In order to mimic the orientations, we first designed three novel collectors to fabricate three nanofibrous layers with those orientations from a polymeric biomaterial in an electrospinning system. Then, we devised a novel direct electrospinning technique to develop a unified trilayered nanofibrous (TN) substrate comprising those oriented layers. The TN substrate supported the growth and orientations of seeded porcine valvular interstitial cells (PVICs) and their deposited collagen fibrils. After one month culture, the obtained trilayered tissue construct (TC) exhibited increased tensile properties over its TN substrate. Most importantly, the developed TC did not show any sign of shrinkage. Gene expression pattern of the PVICs indicated the developing stage of the TC. Their protein expression pattern was quite similar to that of leaflets.

Graphical Abstract

Keywords

trilayered; nanofiber; cardiac valve leaflet; valvular interstitial cell; tissue engineering

*Corresponding author: Jana.Soumen@mayo.edu.

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Disclosure
None

1. Introduction

Cardiac valves control blood flow and allow pressure differentials by opening and closing their leaflets, respectively [1, 2]. Leaflets are primarily composed of valvular interstitial cells (VICs) residing in three apposed layers: circumferentially oriented fibrosa layer, randomly oriented spongiosa layer and radially oriented ventricularis [3–5]. In this trilayered structure, collagen, collagen/proteoglycan and collagen/elastin are the predominant extracellular matrix (ECM) components in these layers, respectively [6]. A diseased or damaged leaflet may result in regurgitation and stenosis affecting blood flow through the heart [5, 7]. These dysfunctionalities are triggered by adverse biochemical factors and mechanical stresses on the residing VICs [8–10].

Surgical replacement of dysfunctional valves with mechanical or bioprosthetic valves is the primary treatment option. However, the risk of thromboembolism from highly durable mechanical valve necessitates lifelong anticoagulation therapy [3, 10, 11]. Owing to leaflet mineralization and mechanical damage, bioprosthetic valves show poor long-term durability [3, 10, 11]. Also, these valves cannot grow or remodel, leading to sequential surgeries in patients, especially pediatric patients.

As an alternative, researchers have been making their efforts to develop heart valves through tissue engineering [12–14]. Typically, tissue engineering of a heart valve occurs by seeding appropriate cells in a synthetic or natural substrate and then culturing the cell-seeded substrate statically in-vitro with the option to apply dynamic physiological environment in a bioreactor or by directly implanting a substrate in the aortic or pulmonary position of a heart [3, 15, 16]. Also, some researchers have fabricated a tissue-engineered valve using cell sheets without requiring any scaffolding material [17, 18]. However, a tissue-engineered leaflet that lack the trilayered-structure of a native leaflet may face major functional problems in long-term valve replacement because long-term efficient leaflet functionalities depend on the appropriate structure of a leaflet [15, 19, 20]. In previous research studies, most of tissue-engineered valve leaflets ended up with shrinkage either during tissue engineering or after implantation in an in-vivo model although they were capable to bear the physiological load and one of the possible reasons of shrinkage could be lack of native trilayered-structure in the tissue-engineered leaflet as none of them had that required native structure [14, 21, 22].

Among different types of substrates such as 3D solid porous substrate, hydrogel-based substrate and fibrous substrate, a fibrous substrate mimics the ECM fibril morphology of a native leaflet. Thus, we opted for fibrous substrates made of nanofibers/microfibers to provide a compatible morphological environment to the culturing cells. However, tissue-engineered leaflets developed with fibrous scaffolds comprising mainly randomly oriented fibers were not successful for their translation to in-vivo models [3, 23]. Thus, we opted for fibrous substrates with a trilayered-structure mimicking the layers' orientations of a native leaflet so as to obtain an oriented trilayered cell-cultured tissue construct (TC) at the end of the process.

Nanofibers can be fabricated through electrospinning, phase separation and self-assembly techniques [24–26]. Among them, electrospinning is most frequently used because it is a versatile technique for preparing nanofibrous substrates. This technique is applicable to most polymers, easy to handle and cost-effective [27, 28]. In this technique, shape and form of the collectors influence the orientations of depositing fibers [29–31]. Thus, circumferentially, randomly and radially oriented nanofibrous layers can be fabricated by developing appropriate collectors. These layers can then be arranged one over another to fabricate a trilayered nanofibrous substrate.

Polycaprolactone (PCL) is commonly used in biomedical applications, especially as an implantable biomaterial [32, 33]. PCL is used to prepare FDA approved drug delivery devices and substrates for tissue engineering and thus, those devices/substrates don't face any restriction for *in vivo* application [29, 30]. It is also easy to electrospin. Its degradation properties depend on its molecular weight and types of applications. PCL polymer with 80 kDa molecular weight (used in this study) has a degradation period up to 3 years [34]. Thus, the substrate fabricated from this polymer will provide support to the developing tissue and its residing cells for a long period of time.

VICs are the main residing cells in a leaflet and take part in regeneration and remodeling of a leaflet. VIC-like fibroblast cells have shown some success in heart valve tissue engineering and VIC-like progenitor cells have taken part in in-vivo regeneration and remodeling of an implanted engineered leaflet [13, 14]. In previous studies, VICs from pig (PVICs) also have been used to study in-vitro heart valve development [15, 23]. Thus, in this study, we opted for PVIC as the seeding cell.

Here we aimed to develop trilayered nanofibrous (TN) substrates that mimic the morphologies of the fibrosa, spongiosa and ventricularis layers of a native leaflet, sequentially, by applying layer-by-layer nanofiber deposition in an electrospinning system. As this study is to understand the behavior of PVICs on a TN substrate and a flat substrate would be easy for uniform cell seeding, culturing and characterization *in vitro*, we opted to develop a flat prototype TN substrate which resembles the top-view projection of three native leaflets together. These TN substrates were seeded with PVICs and then cultured statically for one month and analyzed with respect to mechanical properties, growth and orientations of seeded PVICs and their deposited collagen fibrils and gene expression. In addition, the shrinkage of the cell-culture constructs was examined.

2. Materials and methods

2.1 Fabrication of Electrospinning Collectors

Two aluminum rings of inner diameter and outer diameter 1 and 1.05 inch, respectively and of thickness 0.04 inch were prepared from an aluminum plate. Two holes were made at opposite sides of the periphery of a ring to hold the ring by two rods extended from a stand with a clamp. Another rod was extended from the same stand to the middle of the ring to prepare a ring-with-dot collector in front the spinneret to fabricate a radially oriented layer. The ring without the middle rod was used to prepare a randomly oriented nanofibrous layer. To prepare circumferentially oriented layers, equally spaced 24 holes of 24 gauge diameter

were made on the second aluminum ring. Twelve aluminum spokes made of 24 gauge aluminum wire were placed in the opposite holes of the aluminum ring to prepare spokes-in-ring collector. This collector was held by two rods extended from the stand with a clamp in front of the spinneret.

2.2 Fabrication of Trilayered Substrate

A 9% (wt/v) polycaprolactone (PCL, MW: 80 KD, Sigma Aldrich, USA) solution in trifluoroethanol (Sigma Aldrich, USA) was electrospun at a discharge rate of 0.3 ml/hr, a gap-distance between spinneret needle and collector of 14 cm and a voltage-supply of 15 kV to produce PCL nanofibers with radial orientation on the ring-with-dot collector. Nanofibers were deposited on the inner area of the metal ring as well as on the ring. Obviously, more nanofibers were deposited on the metal ring than on the inner space area of the ring. So, nanofibrous layer on the ring was thicker than the layer in the inner space area of the ring. This thicker peripheral nanofibrous layer worked as a supporting nanofibrous ring-frame to the substrate. Middle rod of the ring-with-dot collector was then removed to electrospin randomly oriented nanofibers over the radially oriented layers. The produced bilayer with peripheral nanofibrous ring-frame was then removed from the metal ring collector and placed on the spokes-in-ring collector and electrospinning was performed. After producing a circumferentially oriented nanofibrous layer on the bilayered substrate, the trilayered nanofibrous (TN) substrate with peripheral nanofibrous ring-frame was removed from the collector. The TN substrate was flat and these final trilayered nanofibrous substrates were used for cell seeding or their characterizations. The thickness of the samples (n=5) at different area (n=3) were recorded with a thickness gauge (Mitutoyo, Japan) and their mean and standard deviation were calculated.

2.3 Tensile Testing

Tensile test was performed following our previously published protocol [35]. In brief, microscale tensile tester (Bose, USA) was used for uniaxial tensile testing of TN substrates and fresh trilayered cell-cultured tissue constructs (TCs). TCs were developed by culturing PVICs in TN substrates for one month in presence of ascorbic acid in culture media. TN substrates and TCs were cut into pieces with 1/3 size of original sample to obtain a shape quite similar to the shape of a porcine aortic valve (PAV) leaflet. Washed TCs were kept in PBS at 4°C. Two hard paper window frames with the window dimensions 11 mm X 10 mm were used to hold a sample and prevent unwanted damage to the sample before testing [35]. A sample strip of 10 mm width was cut from a 1/3-sized sample and was then sandwiched between two window frames and glued (Loctite super glue) to prepare a test sample. The glue was allowed 2 min to dry. TC samples were humidified during that period by pouring a tiny drop of PBS per minute on the middle of the sample. The test sample was then immediately placed in a tensile tester and was loaded at the extension rate 0.1 mm/sec after cutting of the window frame along the cut line. A 500 load cell was used to sense the load on the sample until failure. Within 1 hr of fresh TCs collection, tensile tests were completed to avoid any damage to them. In addition to TC and TN substrate, individual layers of TN substrates were investigated for their tensile properties. TC, TN substrate and circumferentially oriented layer were tested along circumferential direction. Radially oriented layers were tested along radial direction. For randomly oriented layers, any

direction was acceptable. The thickness of the samples was measured using images obtained applying transmission electron microscope or with a thickness gauge. 3 samples of each type were used for tensile testing. Load vs displacement data for each specimen were collected by the tester. Stress and strain data were calculated using thickness, width (10 mm) and length (11 mm) of the specimen. From the stress-strain curve, tangent modulus, yield stress and yield strain for each specimen were measured. Their average values and standard deviations were then calculated.

2.4 Delamination test

The TN substrates ($n = 3$) were placed in an accelerated wear testing (AWT) instrument (TA Instrument, USA) which provided physiological dynamic load (120/80 mm of Hg) with stretching and bending of the substrates but in accelerated manner (frequency > 15 Hz). All samples were run until they were damaged or ruptured. The damaged/ruptured samples were removed from the AWT instrument and checked thoroughly if there were any delamination in the samples. The layered-portion of the damaged/ruptured substrates (without external ring) were removed and placed in an aqueous bath sonicator for 10 min for any delamination of the layers in case they were merely attached.

2.5 Cell Extraction and Culture

Aortic valve leaflets were aseptically collected from pig heart obtained from Hormel Foods (Austin, MN, USA) by opening the heart and then snapping the leaflets from the aortic valve with a sterile scissors. These PAV leaflets were then washed in copious amount of sterile PBS to be used for different purposes including cell extraction. For cell extraction, few PAV leaflets were placed in trypsin (Invitrogen, USA) at 37°C for 5 min. The leaflets were then swabbed gently to remove the endothelial layer from their surfaces and then digested in 0.5% (wt/v) type I collagenase (Worthington Biochemical, Lakewood, NJ) in PBS at 37°C for 5hr. PVICs were then isolated by centrifuging the digestion at 1000 rpm for 10 min and resuspended and expanded in normal tissue culture (NTC) media from Dulbecco's modified Eagle's medium (DMEM, Corning, Corning, NY) supplemented with 10% fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO) and 1% penicillin-streptomycin (Life Technologies, USA) in tissue culture flasks.

2.6 Cell Seeding and Culture

The TN substrates were disinfected by first exposing to UV light for 15 min and then by incubating them in 70% ethanol for 1 hr at room temperature (25°C). The substrates were then washed in copious amount of sterile PBS in a sterile place. PVICs with passage of 3–4 were cultured for 7days in tissue culture flasks before seeding them on TN substrates. 1 million PVICs in 2 ml NTC media with ascorbic acid (150µg/ml) (NTC-A media) were seeded on sterile TN substrates. Ascorbic acid improves collagen deposition. After first day of culture, NTC-A media was replenished to remove the non-attached cells. The seeded cells in the TN substrates were cultured in NTC-A media for one month with replenishing of media every three days to produce TCs.

After 2 days culture, one cell-seeded TN substrate was used to make cell-seeded TN strips ($n=6$). Each cell-seeded TN strip was made by removing the trilayered portion between two

adjacent spokes of a cell-seeded TN substrate. The strips did not have any part of a spoke but had the thick outer nanofibrous ring (partial). The seeded cells in the TN strips were cultured in NTC-A media for one month with replenishing of media every three days.

2.7 Toxicity

1 million PVICs in 2 ml NTC media with ascorbic acid (150µg/ml) (NTC-A media) were seeded on sterile single-layered aligned nanofibrous substrates. Cell seeded substrates were cultured for 14 days and TUNEL assay was performed on them at 1, 3, 7 and 14-day time points. Light microscopy (Nikon, Japan) was applied to image the stained samples. A total of three substrate samples were used for the assay. A large number of images from each sample (layered part only) were collected and analyzed.

2.8 Proliferation

1 million PVICs in 2 ml NTC media with ascorbic acid (150µg/ml) (NTC-A media) were seeded on sterile TN substrates. A total of three TN substrate samples were applied for the test. Seeded substrates were cultured and Alamar Blue assay was performed on them at 1, 3, 7 and 14-day time points. Using a spectrophotometer (SpectraMax Plus 384, Molecular Devices, USA), the absorbance data of the assay solutions were measured. Absorbance data of solutions from known cell were applied to produce a calibrated curve and using this curve, the measured absorbance data of the assay solutions were transformed to cell numbers.

2.9 SEM imaging

TN substrates, TCs and PAV leaflets were processed for SEM imaging. For cross-sectional imaging, the samples were placed in OCT matrix (Thermo Scientific, USA) and then were frozen for their transverse sectioning in a microtome. The section samples and whole samples of TCs and PAV leaflets were fixed in 4% formaldehyde (Sigma Aldrich, St. Louis, MO) overnight at 4°C. The samples were then rinsed in PBS, dehydrated in a graded ethanol series and dried in a critical point drier. Dried samples including TN substrates were sputter coated with gold-palladium at 18 mA for 20 s and imaged with scanning electron microscope (Hitachi, Japan) with EDX detector (Carl Zeiss, Germany) at 15 kV.

Different areas (layered part only) (n=5) of several nanofibrous samples (n=4) were imaged and diameters of 60 nanofibers from those images (n=20) were recorded using ImageJ software and the mean and standard deviation of those diameters was calculated. Similar procedure was used to calculate diameter of collagen fibrils.

2.10 TEM imaging

TCs (layered part only) and PAV leaflet samples were fixed in a mixture of 2.5% glutaraldehyde (Sigma Aldrich, St. Louis, MO) and 4% formaldehyde in PBS for 2 hr, rinsed in PBS, fixed in 1% osmium tetroxide for 1 hr, washed in distilled water and then, dehydrated through graded ethanol. For epoxy resin infiltration, the samples were treated with ethanol-acetone mixture (1:1, by volume) for 10 min, acetone for 10 min, acetone-epoxy resin (2:1, by volume) for 30 min, acetone-epoxy resin (1:1, by volume) for 30 min, acetone-epoxy resin (1:2, by volume) for 30 min and epoxy resin overnight. The samples

were then embedded in fresh epoxy resin and cured overnight at 60°C. The embedded samples were sectioned, collected on copper grids and imaged with transmission electron microscopy (Jeol, Japan).

2.11 Protein quantification

To quantify collagen contents in TCs and PAV leaflets, their wet samples were homogenized in water and then hydrolyzed in 37% HCL at 120°C for 3 h. Collagen assay kit (Sigma Aldrich, USA) was used to quantify the collagen content applying company protocol. 50 µl of the hydrolyzed sample was transferred to 96-well plate and dried at 60°C. 100 µl chloramine T/oxidation buffer mixture (94:6) was added to the dried sample in well plate and incubated at room temp for 5 min. 100 µl of diluted DMB reagent was added to the sample and incubated at 60°C for 90 min. After cooling the samples to room temperature, their absorbance at 557 nm were recorded with SpectraMax Plus 384 microplate reader (Molecular Device, USA) and compared with standard curve obtained by measuring the absorbance of known hydroxyproline solution.

To quantify glycosaminoglycan (GAG) contents in TCs and PAV leaflets, their lyophilized samples were digested in papainase buffer containing papain type III (Worthington Biochemical, USA). Supernatants were collected by centrifugation. GAG assay kit (Blyscan, Biocolor, USA) was used to quantify the GAG applying company protocol. In brief, 50 µl DI water was added to 50 µl of the papain digest and then 1 ml GAG dye was added to the solution. The dye mixed solution was placed in a shaker for 30 min and then centrifuged at 12000 rpm for 10 min. after removing the supernatant, the precipitate was dissociated with 0.5 ml dissociation agent. The sample was analyzed at 656 nm absorbance and compared with standard curve obtained by measuring the absorbance of known GAG standard solutions.

Elastin assay kit (Fastin, Biocolor, USA) was used to quantify the elastin in TCs and PAV leaflet samples. Their lyophilized samples were incubated in 0.25M oxalic acid at 100°C for 1 hr. After centrifuging, the supernatant was added to elastin precipitate agent and the precipitate was dyed with dying agent through complete mixing. Through centrifuging, dyed elation was collected as precipitate and then dissociate with dissociating agent. The sample was then analyzed at 513 nm absorbance and compared with standard curve obtained by measuring the absorbance of known elastin standard solutions.

2.12 Gene expression analysis

RNA from TCs and PAV leaflet samples was extracted using Pure Link RNA mini kit (Ambion, Life Technologies, USA) and its supplied protocol. The samples were washed with PBS, snap freezed in liquid nitrogen and kept at -80 °C. The frozen samples were homogenized instantly (thawing occurred at this point) in lysis buffer. The extracted RNA was purified with DNase I (Life technologies, USA) according to manufacturer's protocol. First-strand cDNA was synthesized from purified RNA using High-Capacity cDNA Reverse Transcription kit with RNase Inhibitor (Applied Biosystems, USA). cDNA transcripts were then probed with TaqMan assays for vimentin (Ss04330801_gH), α -smooth muscle actin (α -SMA, Ss04245588_m1), and type I collagen (COL1A1, Ss03373341_g1) using

Lightcycler 480 Probe master mix (Roche, USA). Thermocycling was performed in a Lightcycler 480 (Roche Applied Science, USA) with the following conditions: pre-incubation (95°C, 15 min), 40 cycles of amplification (denaturation: 95°C, 15 s; annealing: 60°C, 1 min; and extension: 72°C, 1 s) and cooling (40°C, 30 s). Target gene data were normalized against ACTB level and analyzed using the comparative cycle threshold (Ct) method.

2.13 Masson's trichrome staining

Fibrosa and ventricularis layer sections were cut from frozen PAV leaflet tissue samples in OCT matrix with a microtome. Tissue sections were on the glass slides while whole TC samples were stand alone. Tissue sections and whole TC samples were fixed in 4% formaldehyde overnight at 4°C. The samples were then rinsed in PBS and then stained with Masson's trichrome using manufacturer's instruction (Sigma Aldrich, USA). The stained samples were then rinsed, dehydrated, mounted on glass slides using a mounting media, and imaged (layered part only) in an optical microscope (Evos, Life Technologies, USA).

2.14 Immunostaining

Fibrosa, spongiosa and ventricularis layer sections were cut from frozen PAV leaflet tissue samples with OCT matrix in a microtome. Tissue sections were on the glass slides while whole TC samples were standalone (were not placed on glass slide). Tissue sections and whole TC samples were fixed in 4% methanol-free formaldehyde (Thermo Scientific, USA) overnight at 4°C. The samples were washed in PBS, incubated in 0.1% Triton X-100 (Sigma Alrich, USA) for 2 min, washed in PBS, and incubated in 10% goat serum (Abcam, USA) for 30 min. The samples were then incubated in a mouse anti-vimentin IgM primary antibody (Novus Biologicals, USA) at a 1:500 dilution in PBS for 1 hr. They were then washed in PBS and incubated in Alexa Fluor 488 (Life Technologies, USA) conjugated goat anti-mouse IgM secondary antibody (Abcam, USA) at 1:500 dilution in PBS for 45 min. The samples were then washed in PBS and mounted on glass slide using Prolong Gold Antifade reagent with DAPI (Invitrogen, USA) mounting media. For smooth muscle actin staining, similar procedure mentioned above was followed except the primary and secondary antibodies were mouse anti-human smooth muscle actin IgG (Dako, USA) and Alexa Fluor 594 (Life Technologies, USA) conjugated goat anti-mouse IgG (Abcam, USA), respectively. The samples were imaged with a confocal laser scanning microscopy (Carl Zeiss, Germany). While stained PAV leaflet samples were on glass slides, stained standalone TC samples were in PBS buffer and thus, stained TC samples were imaged from both sides (layered part only).

α -SMA signal in the immunostained images was quantified using MetaMorph software and ImageJ software. First, percentage of area of the α -SMA signal with respect to the total area of a stained image was measured using MetaMorph software. Total cell number in that image was counted using ImageJ software. α -SMA signal in that image was then calculated per hundred cells using percentage area and total cell count per image data. A total of 7 images were used to obtain an average α -SMA signal for each layer.

2.15 Statistical Analysis

Data from nanofiber diameter, tensile test, protein fibril diameter and protein quantification are reported as mean $\pm$ standard deviation (SD) ($n>3$). For tensile test and gene expression analysis, data from respective items of two groups (TN and TC) are compared using unpaired t-test for statistical analysis. Data were compared using one-way ANOVA followed by Duncan's posthoc tests for statistical significance through GraphPad Prism software. P values <0.05 were regarded to indicate significance.

3. Results and discussion

Morphology and mechanical characteristics associated with the tissue structure define the behavior and functionality of the tissue [15, 30, 36]. In a complex tissue structure with interconnected layers, behavior of the cells relies on the efficient and cooperative structural contribution of each layer. A trilayered leaflet comprising circumferentially oriented fibrosa layer, randomly oriented spongiosa layer and radially oriented ventricularis in a heart valve is such an example (Figure 1a) [3]. In its tissue engineering, development of a trilayered structure with appropriate morphologies is the crucial step. Previous studies used various substrate systems including trilayered hydrogel, trilayered microfabricated/nanofibrous substrates and hybrid nanofibrous substrates to develop an aortic valve leaflet [15, 20, 37]. Nanofibrous substrates showed better results compared to hydrogel because nanofibers mimic the morphology of ECM. These examples emphasize the significance of nanofibers in substrate design for leaflet tissue engineering. In this study, we developed a flat TN substrate mimicking the morphologies of three layers of an aortic valve leaflet, i.e., circumferentially oriented fibrosa layer, randomly oriented spongiosa layer and radially oriented ventricularis layer to observe the behavior of the cultured PVICs.

3.1 Fabrication of trilayered nanofibrous substrate

Three nanofibrous layers were electrospun sequentially to fabricate a flat TN substrate that mimicked the morphological structure of a native aortic valve leaflet (Figure 1b). This flat TN substrate is the top-view projection of three native leaflets together. Fabrication of this TN substrate was completely novel and an important part of this study; thus, we describe the fabrication process here in brief with appropriate images. First, two aluminum collectors of same dimension were designed to fabricate three nanofibrous layers. To produce radially oriented nanofibers, we used a ring-with-dot collector consisting of a ring and a tiny metallic dot at its center (Figure 2a, front view). The collector was held by two rods extended from a clamp (Figure 2b, side view), and a third rod was held axially at the center of the ring. Without this third rod, the ring collector was used to produce randomly oriented nanofibers (Figure 2c, front view; Figure 2d, side view). A spokes-in-ring collector consisting of a ring and 12 spokes was used to produce circumferentially oriented fibers (Figure 2e, front view). It was held by two rods extending from a clamp (Figure 2f, side view). All the collector-setups were electrically grounded before electrospinning.

A 9% (wt/v) PCL solution was electrospun on ring-with-dot collector to produce radially oriented nanofibers (Figures 2g–h). Between the dot and the ring, a specially-formed magnetic field pulled one end of a depositing nanofiber toward the dot and another end

toward the ring radially; thus, deposited fibers were radially oriented [29]. The radially oriented nanofibrous layer had the nanofibrous ring-frame (described in method section in detail) to hold the radial nanofibers inside. The middle rod of the above collector was removed to fabricate randomly oriented nanofibers. In general, a metal plate is used to produce randomly oriented nanofibrous substrate. However, in this study, to make the layer highly porous, we made a randomly oriented nanofibrous layer without any metal plate at the back of the ring collector (Figures 2i–j). After electrospinning, we detached the bilayer substrate from the ring collector and placed it on the spokes-in-ring collector. Electrospinning produced a circumferentially oriented nanofibrous layer (Figures 2k–l) on the bilayer substrate, i.e., a flat TN substrate with three nanofiber-orientations (radial, random and circumferential, sequentially) (Figure 2m). Between the spokes, a specially-formed magnetic field pulled two ends of depositing nanofibers toward two consecutive spokes perpendicularly, so deposited fibers were concentric. Deposited nanofibers on the metal spokes formed nanofibrous spokes (Figure 2l), which kept the circumferentially oriented fibers intact after removal of the TN substrate from the collector. Diameter of nanofibers in the substrates was 340 ± 87 nm (Figures 2n–p). Contrary to other membrane-based nanofibrous substrates used in tissue engineering, the TN substrates were fully standalone due to presence of the thick nanofibrous ring-frame at their periphery (Figure 3). Thus, there was no need for an underlying platform (generally coverslip) that could influence the culturing PVICs in the TN substrates [30, 38]. However, they were very pliable as shown from different angles in Figure 3. Thickness of TN substrates depended on electrospinning time and thus, can be made up to the requirement in future. TN substrates (only layered portion excluding spokes and external ring) developed in this study had a thickness of 145 ± 5 μm with sufficient pore size (>10 μm) (Figure. 2n–p) for cell penetration into the substrates. Each layer had nearly identical thickness that was greater than the size of a cell (~ 10 μm) to provide a 3D environment to the seeded cell.

3.2 Tensile properties of trilayered nanofibrous substrate

In addition to morphology, mechanical properties of a substrate influence cellular adhesion, alignment, migration and growth, and functionality of the construct [36, 39]. Uniaxial tensile testing on TN substrates was performed along their circumferential direction to determine their tensile stiffness and yield stress following our previously used procedure reported elsewhere [35, 40]. In brief, a third of a TN substrate, which was almost equivalent to a native leaflet in shape and oriented layers, was used to make a tensile test specimen (Figures 4a–b). A sample strip (shown by dash lines in figure 4b) was cut from the 1/3-sized substrate sample and then sandwiched between two paper windows. The sample strip was comprised of mainly spokes and trilayered nanofibers between the adjacent spokes. The thick outer ring portions did not exist in the window area and if any thick outer ring (partly) existed, they were in the grip area. The spokes in the sample strip were completely separate from each other and they were connected by the trilayered nanofibers. Further, spokes were much stronger than the trilayered nanofibers between the adjacent spokes. Thus, neither the outer ring nor the spokes influenced the tensile tests of the samples along circumferential direction. All the specimens inserted in the tensile tester had similar alignment. According to ASTM D638 standard, a dumbbell-shaped specimen is generally used for tensile test [41]; however, a dumbbell-shaped specimen cut from a TN substrate could not maintain its

integrity impeccably. Further, previous studies reported the tensile testing of rectangle-shaped nanofibrous specimens [38, 42]. We investigated bulk tensile properties of TN substrates and individual layers of TN substrates. Circumferential layers were tested along circumferential direction. Radial layers were tested along radial direction. For random layers, uniaxial tensile test at any direction was similar. Test results of TN substrates and their individual layers are listed in Table 1. Measurement of tensile properties of a TN substrate in radial direction could not provide the actual results due to presence of thick nanofibrous spokes in the circumferential layer of the substrate and thus, measurement of its biaxial tensile properties could not be worthy. In fact, in radial direction, a TN substrate had spokes and the distance between two spokes narrowed toward the center. If a sample strip without a single spoke were cut from a substrate to prepare a test sample for a tensile test in radial direction, the sample would not be wide enough and sample would be too fragile to run the test. In case of wider strip, spoke would exist in the strip; tensile properties of spokes would be part of the obtained results and thus, would not be the true tensile properties of only three layers in a substrate in radial direction.

Tensile tests of TN substrates showed a typical stress-strain profile of a fibrous sample (Figure 4c). The bulk tensile modulus, yield stress (stress at which the substrate or TC deformed plastically) and yield strain (strain at yield stress) of TN substrates were 0.79 ± 0.18 MPa, 0.39 ± 0.10 MPa and $60 \pm 18\%$, respectively. It is to note that the tensile stiffness and yield stress of radial layer in radial direction were higher than those of the circumferential layer in circumferential direction. It is to note that higher standard deviation was observed in tensile test results from circumferential layer compared to that from radial and random layers due to complex form of its collector. The more the complexity is, the more non-uniform the electrostatic force is between the different regions of the collector and the spinneret. Thus, fiber deposition density on a circumferential collector varied more compared to that on other collectors and thus, the standard deviation was higher for circumferential layer compared to other layers.

In a native leaflet, the mechanical properties in the radial direction are lower than that in the circumferential direction because radial layer is consisted of mainly elastin whereas circumferential layer is consisted of comparatively stronger material – collagen [43]. Conversely, all the layers in a TN substrate were made of PCL nanofibers. In the radial layer, nanofibers were straight, while in the circumferential layer, they were circumferentially oriented. Due to this curvilinear orientation, tensile properties of nanofibers in the circumferential layer were lower compared to that in the radial layer. A detailed explanation on this variation of tensile properties of different nanofibrous layers can be found in our previous paper [40].

To achieve the mechanical properties of three layers of a TN substrate proportionate to that of the respective layers of a native leaflet, another polymeric material (example: polyglycerol sebacate (PGS) – a comparatively softer material) can be used [15]. The mechanical properties of PGS depend on its crosslinking density, so its mechanical properties can be adjusted. Further, PGS can make a good composite polymer with PCL [15]. Thus, appropriate polymer (s) can be selected from this PGS, PGS/PCL and PCL

polymer set to make nanofibrous layers with mechanical properties proportionate to that of native leaflet layers.

3.3 Delamination test

In the above-mentioned tensile test of TN substrates along the circumferential direction, we did not observe any delamination of layers in a TN substrate, although different layers had different bulk tensile properties. To confirm further that layers in the TN substrates would not delaminate in real application, physiologic dynamic load (120/80 mm of Hg) was applied on the TN substrates ($n = 3$) in an accelerated wear testing (AWT) instrument until failure ($>70,000$ cycle) (Figures 5a and 5b).

In the AWT system, the fluid could not flow through the flat TN substrate as happens in aortic heart valve. Instead, this substrate expanded radially due to fluid pressure in the first half of a cycle (systole), then it expanded radially in the opposite direction in next half of the cycle (diastole), also due to fluid pressure in opposite direction and these phenomena occurred repeatedly. Thus, if there was no unification among the layers, layers should have delaminated as bulk mechanical properties of each layer were different leading to dissimilar responses from three layers individually in the substrate. This was the principle of this AWT study. This AWT test of the TN substrate was not comparable to the AWT testing of an aortic valve either bioprosthetic or mechanical. The TN substrate was much weaker than an aortic valve leaflet and that is why, the TN substrate could not sustain long time in the AWT system.

The samples were ruptured cross-sectionally as observed in Figure 5c. Ruptured sections were removed from samples and placed in bath sonicator to determine whether any delamination occurred during physiological loading in AWT. However, the layers in the ruptured sections were intact and firmly attached to each other as they were before any testing (Figure 5d). Due to the unification of layers in a substrate, each layer contributed to load bearing during tensile testing of the TN substrate in circumferential direction and tensile stiffness and yield stress of a TN substrate were higher than that of circumferential layer (Table 1).

3.4 Toxicity and proliferation

PCL-based biomedical devices face no problem to obtain FDA approval. However, a toxic chemical, trifluoroethanol, was used to prepare PCL nanofibers. Although, the prepared nanofibrous substrates were sterilized through exposing to UV light, incubating in 70% ethanol and then washing in copious amount of PBS in sterile environment before cell seeding, a very small residue of TEF in the substrates could be toxic. It is to note that 15 min UV exposure to TN substrates did not change any physico-chemical and bioresponsive properties of the substrates [44]. TUNEL staining assay was performed on the cell-cultured substrates to detect any toxicity of the prepared nanofibers. The substrates prepared for this toxicity study were one-layer aligned nanofibrous substrates (for easy visualization of cells). Cell death was around 6–7% of total the population (Figure 6a). Similar percentage of cell death generally occurs during general cell culturing in tissue culture flask. Thus, the

developed PCL trilayered nanofibrous substrates were non-toxic for cell culture and tissue engineering.

To further prove that TN substrates were not toxic and they were worthy for cell growth, cell proliferation measurements were performed at 1, 3, 7 and 14-day time points. Cells proliferated well on the TN substrates with passage of time (Figure 6b). At Day 1, the counted cell number was around 723k, which suggested that all seeded cells did not attach to the substrates. After 14 days of culture, cell number rose to ~ 5 million. Further, number of cells at any time point was significantly higher compared to the previous time point(s). These results indicate that TN substrates made from PCL polymer were non-toxic and biocompatible for cell growth.

3.5 Shrinkage of the cell-cultured construct

Contraction of cells in a tissue engineered construct intended for biologic heart valve fabrication is not welcome at all. Once contraction of cells started, the tissue construct would shrink further over time leading to regurgitation, stenosis and other adversaries in the developed heart valve construct [45]. In this study, VICs from PAV leaflets were seeded on TN substrates and individual layers for one month. The constructs developed with TN substrates (stiffness: 0.79 MPa) did not show any shrinkage after one month of culturing (Figure 7a). However, we observed shrinkage of several individual random layer substrates (stiffness: 0.89 MPa) and radial layer substrates (stiffness: 1.13 MPa) except circumferential layer substrates (stiffness: 0.33 MPa) (Figure 7b-d). It is to note that all three individual layer substrates and a TN substrate had thick outer nanofibrous ring. So, thick outer nanofibrous ring could not obstruct the shrinkage of the substrates caused by contraction of PVICs. However, it can be speculated that thick spokes present in the TN substrate and individual circumferential layer substrate prevented the shrinkage of substrates under contraction of the cells. To negate the speculation, we seeded the PVICs on several whole TN substrates and statically cultured for two days for complete adhesion of cells to the substrates. Then, we cut several strips (n=6) between several two adjacent spokes of the cell-seeded substrates and cultured the strips for one month in NTC-A media. The strips did not have any spokes but had a very small portion of thick outer nanofibrous ring required for handling the strips. The thickness of the outer ring in a strip was higher compared to that of the outer ring in an individual layer. It was possible that the thicker outer ring in a whole TN substrate prohibited its shrinkage. Hence, shrinkage assay was performed on TC strip. The outer ring was at one side of the strip and another side of the strip was completely free from outer ring. If the cultured PVIC contracted, then free-side of the TC should have contracted. After one month culture, none of the strips showed any shrinkage i.e. PVICs did not contract in any of the six strip-samples (Figure 7e). So, thicker outer ring did not prohibit the shrinkage of the whole TN substrate when PVICs were cultured on it. It is to note that after one month culture, not all individual radial layer and random layer substrates showed shrinkage i.e. PVICs did not contract in all individual radial layer and random layer substrates. Non-uniform nanofiber deposition, non-uniform cell density in the substrates and other unknown factors could be the reasons which need further investigations to identify them. Further, more individual radial layer substrates shrank compared to individual random layer substrates. However, not a single individual circumferential layer showed any

shrinkage and low mechanical properties of this layer could be the reason. To study the effect of higher mechanical properties of a substrate on contraction of PVICs, PVICs were cultured on polystyrene coverslips (stiffness = $\sim 3\text{GPa}$) and within 10 days of culturing, PVICs contracted leading to bending of the coverslips (Figure 7f). Conversely, Wang et al. cultured PVICs on a light-sensitive hydrogel with changeable stiffness and found that PVICs showed myofibroblast phenotype (one of the signs for contraction of VICs) when the stiffness of the hydrogel was increased to as low as 8kPa [46]. A TN substrate had three native orientations and its cumulative mechanical properties were less than those of individual radial and random layer substrates but higher than those of individual circumferential layer substrates. Thus, in addition to higher mechanical properties, lack of native orientations and morphologies in the substrates could have caused the contraction of PVICs leading to shrinkage of their respective cell-cultured constructs.

3.6 Morphologies of seeded cells and deposited collagen

As the TN substrates had sufficient pore size for cell penetration into the substrate, PVICs were present throughout the substrates. After one month culturing in NTC-A media (normal tissue culture media with ascorbic acid), we characterized the obtained constructs. Regarding morphology, PVICs at the outer layers (circumferential and radial layers) of the constructs were stretched, spindle shaped, and oriented along the nanofibers of the respective layers (Figures 8a–b). Their morphologies and orientations were comparable to those of PVICs in fibrosa and ventricularis layers of native aortic valve leaflets (Figures 8c–d). Instead of SEM images, immunostained images of native leaflets were useful to detect cells within numerous collagen fibrils. At higher magnification, a large number of aligned collagen fibrils were observed in the circumferentially and radially oriented layers of the TCs (Figures 8e–f). The orientations of the collagen fibrils in those layers were similar to the orientations of collagen fibrils in fibrosa and ventricularis layers of a native leaflet (Figures 8g–h). Collagen nature of these fibrils in TCs and native leaflets was confirmed by Masson's trichrome staining (Figures 8i–l). Due to more cells in tissue-engineered constructs compared to native construct, red-stained cells (cytoplasm) against blue-stained collagen fibrils brought violet color in the former. Appropriate alignment of collagen fibrils in the layers of TN substrates indicated that nanofibers present in the substrates guided the PVICs to deposit aligned fibrils vital for proper functionality of the construct [47, 48]. Collagen fibril diameter ($24 \pm 4\text{ nm}$) in the TCs was less than that ($\sim 35\text{ nm}$) in native leaflets. Thus, TCs were possibly in growing stage [49, 50]. After one month of culture, the thickness of TCs was $\sim 185\text{ }\mu\text{m}$ and therefore, there was an increment of thickness of TN substrate samples by $\sim 40\text{ }\mu\text{m}$ (originally: $\sim 145\text{ }\mu\text{m}$) due to deposition of ECM proteins. Thickness of TN substrates can be increased in future per requirement as it depends on electrospinning time. However, electrospinning parameters and polymer solution concentration needs to be changed to keep the pore size sufficient enough for cell penetration into the thick substrate [51].

Three layers — circumferential (x), random (y) and radial (z) — were detected distinctly in the cross-sectional images of the constructs obtained through a transmission electron microscope (TEM) (Figure 9a). Shape, size and orientation of PVICs in these layers of a TC were also distinct due to diverse orientations of nanofibers present in the layers. Cells were

aligned and elongated along the nanofibers in the circumferential layer (Figure 9b). They had assorted shapes and elongations in the random layer (Figure 9c). In the radial layer (orientation perpendicular to the paper), the cells were either circular or elliptical — the shape of the cell cross-section (Figure 9d).

3.7 Tensile properties of trilayered construct

To determine and compare bulk tensile properties of TCs (Figure 4d) to that of TN substrates, we performed uniaxial tensile tests on TCs along the circumferential direction (Figure 4b). Detailed protocol of tensile test method is available in our previously published paper [35]. After one month culture, the tensile curve profile of the obtained trilayered tissue constructs (TCs) exhibited an increase in tensile properties over their TN substrates (Figure 4c). Biaxial tensile properties of the construct were not measured because presence of thick nanofibrous spokes in circumferential layer would not provide meaningful results (discussed before).

The bulk tensile modulus of TCs was 2.50 ± 0.67 MPa which was higher than that (0.79 ± 0.18 MPa) of TN substrates. Bulk yield stress of TCs was 0.86 ± 0.22 MPa which was higher than that (0.39 ± 0.10 MPa) of TN substrates. The modulus and yield stress of TCs were significantly higher than those of TN substrates, respectively, ($p < 0.05$, by unpaired t-test). Although tissue construct with higher mechanical properties (tensile modulus: ~ 3.49 MPa) are required for an implantable heart valve development, this result shows the positivity of the trilayered structure in tissue engineered leaflet development [16, 52]. Larger time point and dynamic environment may improve the mechanical properties of the construct [12, 16, 53].

3.8 Analysis on deposition of proteins at different layers of a construct

Functionality of the leaflets in a heart valve is associated with the characteristics of ECM proteins, especially collagen, proteoglycan and elastin; hence, presence of these proteins in an engineered construct is crucial and will delineate the efficiency of the construct [3, 10]. In the cross-sectional images of the constructs obtained through a transmission electron microscope (TEM), presence of collagen was distinctly detected. As observed in native leaflets, three distinct layers (layers x, y and z separated by white lines) containing major protein — collagen — were seen in the TCs (Figures 10a–b). In the ventricularis layer of a leaflet and its equivalent radial layer of the TCs, collagen fibrils were radially oriented (perpendicular to the paper) (Figures 10c–d). The spongiosa layer of a native leaflet and its equivalent random layer in the TCs were less dense compared to the remaining two layers (Figures 10e–f). Collagen fibrils were randomly oriented in the layer of the TCs as seen in the spongiosa layer of a native leaflet. Many highly aligned collagen fibrils were observed in circumferential layer of the TCs and in a native fibrosa layer (Figures 10g–h). These observations lead to a conclusion that the orientations of collagen fibrils in all layers of the TCs were similar to those in the corresponding layers of a native leaflet and presence of oriented collagen fibrils is very important for the success in tissue-engineered heart valve development [15, 54, 55].

In addition to structural and qualitative characterization, we measured relative quantities (with respect to dry weight of leaflet and TC) of collagen in native leaflets and in TCs. We also measured relative quantities of proteoglycan and elastin in them. Collagen deposition in TCs was 129 ± 12 $\mu\text{g}/\text{mg}$ (in terms of hydroxyproline), compared to 153 ± 9 $\mu\text{g}/\text{mg}$ in native leaflets. Proteoglycan was 5 ± 1 $\mu\text{g}/\text{mg}$ in the constructs and 5 $\mu\text{g}/\text{mg}$ in the leaflets. Elastin deposition was 67 ± 10 $\mu\text{g}/\text{mg}$ in TCs and that in leaflets was 74 ± 10 $\mu\text{g}/\text{mg}$. Overall, the relative quantities of collagen, proteoglycan and elastin in native leaflets and TCs were quite close, although TCs were in developing stage in vitro and native collagen-GAG-elastin structure was not observed in the TCs, which is bit surprising; however, other researchers have reported similar findings in their studies [15, 55]. Reports also indicate that substrate morphology and its mechanical properties affect the cell adhesion, cell–matrix and cell–cell interactions, which in turn influence protein deposition [37, 56]. Mimicked nanofiber orientations with pore interconnectivity in substrate system might have provided a favorable environment for sufficient ECM deposition.

In a native leaflet, presence of collagen is observed mostly in fibrosa layer. In the spongiosa layer and ventricularis layer, presence of mostly GAGs and elastin are observed, respectively. In TC samples, presence of collagen, GAG and elastin was established through protein quantification assays. Although all three protein materials were present, they were not layer-specific as we see in a native leaflet and thus, it can be speculated that nanofiber orientation was not able to influence the deposition of specific ECM components in specific layers as observed in native leaflets.

3.9 Gene expression and immunostaining analysis

At the developing and remodeling stages of valve leaflets, PVICs show active myofibroblast phenotype, characterized by high gene expression of vimentin (fibroblasts) and α -smooth muscle actin (smooth muscle cells) [57]. PVICs in both TCs and native leaflets showed high expression of vimentin (Figure 11a). However, their α -SMA expression varied; α -SMA expression in TCs was almost two and half times that in native leaflets (they were significantly different, $p < 0.05$ by unpaired t-test). High α -SMA expression of PVICs in TCs confirms that PVICs had growth-prone active myofibroblast phenotype as TCs were in developing stage. PVICs in both native leaflets and TCs had almost similar COL1A1 expression.

To ascertain the above gene expression, we performed immunostaining native leaflets and TC specimens with antivimentin and anti α -SMA markers. We visually observed least and highest α -SMA staining in the circumferential and radial layers, respectively; α -SMA staining in the random layer was in between (Figure 11b). To confirm the above observation from visual inspection, α -SMA signals on three layers (circumferential, random and radial) were quantified as shown in Figure 11c. α -SMA signal in radial layer was the highest and in circumferential layer was the lowest and their difference was statistically significant (by Student's t-test, $n = 7$). This staining signal quantified data perfectly corresponds to the tensile properties of the layers (Table 1). All the layers were made of nanofibers from PCL polymer applying similar parameters. So, all the fibers in the layers had similar properties except mechanical properties and orientations. However, for the PVIC, orientations and

morphologies in the layers were not incompatible as they exist in a native leaflet. Therefore, it is possible that mechanical properties of the layers could have been responsible for the cells behaving differently. Higher mechanical properties of a substrate may have caused higher α -SMA expression of PVICs, which is unfavorable (may cause contraction) for heart valve development [40, 46, 58]. Growth of tissue construct also can be responsible for higher α -SMA expression by PVICs [59]. It is mentioned before that PVIC-cultured individual circumferential layer (tensile modulus: 0.33 MPa) did not show any shrinkage whereas PVIC-cultured individual random (tensile modulus: 0.89 MPa) and radial (tensile modulus: 1.13 MPa) layers showed some shrinkages and these observations may be related to higher α -SMA expression of PVICs in different layers. Vimentin-stained PVICs were observed in both the TCs and native leaflets, endorsing their fibroblast phenotype (Figure 11b). In general, PVICs in a healthy leaflet show quiescent fibroblast characterized by moderate or high vimentin expression and very low α -SMA expression [57].

Ultimate goal is to develop a tissue engineered heart valve especially its leaflets that should not shrink during development or afterwards. In this respect, behaviors of PVICs cultured on the flat trilayered nanofibrous substrate have brought a very relevant point in leaflet development through tissue engineering. Trilayered structure with orientations exist in a native leaflet would be the best option for leaflet substrate designing. Single orientation or no orientation is likely to cause contraction of the cells in the developed construct although individual circumferential layer did not shrink possibly due to low mechanical properties as in real case, the circumferential layer would have higher mechanical properties. Also, some extra other points can be pulled out from this study for substrate fabrication and in vitro tissue engineering. Three nanofibrous layers from more than one polymeric material would be worthy to bring the mechanical properties proportionate to that exist in a native leaflet. Direct deposition of nanofibers would benefit in developing a unified substrate consisted of different layers. One month static in vitro cell culture may not be enough to develop a construct that can be implanted in vivo directly.

4. Conclusions

Through electrospinning, we developed a trilayered nanofibrous substrate mimicking the morphologies of three layers of a native heart valve leaflet. Growth of culturing PVICs in the substrate was continuous and a trilayered constructs was developed. Orientations and morphologies of one-month cultured PVICs and their deposited collagen fibrils in each layer of the construct were comparable to those in native leaflets. Tensile properties of the trilayered construct were higher than those of the polymeric TN substrate. Besides collagen, presence of GAG and elastin in the construct were quite equivalent to that in a native leaflet. Comparable expressions of vimentin and COL1A1 gene and higher expression of α -SMA by PVICs in the construct with respect to a native leaflet confirmed the growing stage of the construct. Most importantly, the construct did not show any shrinkage after one month of PVIC culturing – possibly due to mimicked trilayered structure of the substrate. This study builds a foundation for our future endeavor to develop a leaflet-shape trilayered nanofibrous substrate and its tissue-engineered leaflet construct.

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Statement of Significance

This manuscript mainly talks about development of a novel trilayered nanofibrous substrate mimicking the morphologies of a heart valve leaflet. Then it describes the culturing of valvular interstitial cells that reside in the leaflet, in the substrate and compares the behavior of the cultured cells with that in the native leaflets in terms cell morphology, protein deposition and its orientation, and molecular signature. This study builds the groundwork for our future trilayered, tissue-engineered leaflet development. This research article would be of great interest to investigators and researchers in cardiovascular tissue engineering especially in cardiac valve tissue engineering through biomaterial-based tissue engineering.

Figure 1.

Schematic structures of an aortic valve leaflet and a synthetic trilayered substrate. (a) The aortic valve leaflet is consisted of a circumferentially oriented fibrosa layer, a randomly oriented spongiosa layer and a radially oriented ventricularis layer (left) and their top-view projections (right). (b) Mimicking the structure of the trilayered leaflet, a trilayered nanofibrous substrate was fabricated by superimposing a circumferentially oriented nanofibrous layer, a randomly oriented nanofibrous layer and a radially oriented nanofibrous layer

Figure 2.

Fabrication of trilayered nanofibrous substrates through electrospinning and their characterizations. (a) Photograph of a ring-with-dot collector (front view), Scale: 0.40 cm. (b) Photograph of a ring-with-dot collector held by two metal rods extended from a clamp (side view). Another rod extended from the clamp was used to hold the tiny sphere at the center of the ring (side view), Scale: 0.60 cm. (c) Photograph of a ring collector (front view), Scale: 0.40 cm. (d) Photograph of a ring collector held by two metal rods extended from a clamp (side view), Scale: 0.60 cm. (e) Photograph of a spokes-in-ring collector (front view), Scale: 0.40 cm. (f) Photograph of a spokes-in-ring collector held by two rods extended from a clamp (side view), Scale: 0.60 cm. (g) Photograph of the radially oriented nanofibrous substrate, Scale: 0.50 cm. (h) SEM image of nanofibers in the radially oriented nanofibrous substrate, Scale: 650 μm . (i) Photograph of the randomly oriented nanofibrous substrate, scale: 0.50 cm. (j) SEM image of nanofibers in the randomly oriented nanofibrous substrate,

Scale: 650 μm . (k) Photograph of the circumferentially oriented nanofibrous substrate, scale: 0.50 cm. (l) SEM image of nanofibers in the circumferentially oriented nanofibrous substrate, Scale: 650 μm . (m) Photograph of the trilayered nanofibrous substrate containing circumferentially, randomly and radially oriented nanofibrous layers, scale: 0.50 cm. (n) SEM image of nanofibers in the radially oriented layer, scale: 2.5 μm . (o) SEM image of nanofibers in the randomly oriented layer, scale: 2 μm . (p) SEM image of nanofibers in the circumferentially oriented layer, scale: 5 μm . Two-headed white arrows show nanofiber orientations in respective layers

Figure 3. Standalone and pliable TN substrate shown from different angles. It bends with very little pressure between two fingers

Figure 4.

Tensile tests of TN substrates and TCs. (a–b) Photographs of (a) a PAV leaflet and (b) a third of a TN substrate used for tensile tests. A third of a TN substrate was almost equivalent to a leaflet in terms of shape and layer orientations. Arrows indicate the direction of tensile loading. Dotted lines show the sample strip cut from the 1/3-sized sample was used for tensile tests. (c) Tensile stress-strain profiles of a TN substrates and a TC. (d) Photograph of a TC soaked in PBS on a glass slide, scale: 4.5 mm

Figure 5.

Delamination experiments on TN substrates (n=3) in an accelerated wear testing (AWT) instrument. (a) Graph of AWT dynamic load (120/80 mm of Hg) that was applied on the TC sample during testing. (b) A TN substrate sample placed in AWT instrument. (c) Damaged sample after testing for more than 70000 cycles. (d) Intact central part of the damaged TN substrate (c) after 10 min sonication in a bath sonicator

Figure 6.

Survival of VICs on aligned monolayer PCL nanofibrous substrates and proliferation of PVICs on PCL TN nanofibrous substrates over a 14-day period assessed by the TUNEL and Alamar Blue assays, respectively. (a) Survival test data were presented in terms of fraction of total number of cells. Data refer to mean value $\pm$ standard deviation ($p < 0.05$, by Student's t-test, $n = 3$, not statistically significant). (b) Proliferation test data refer to mean value $\pm$ standard deviation (* $p < 0.05$, by Student's t-test, $n = 3$, two numbers bracketed were statistically significantly different)

Figure 7. Contraction of PVICs on different substrates. (a) TN substrate. (b) Circumferential layer substrate. (c) Random layer substrate. (d) Radial layer substrate. (e) TN-strip substrate. (f) Polystyrene coverslip

Figure 8.

Characteristics of cultured PVICs and deposited collagen fibrils in TCs and PAV leaflets. (a–b) SEM images of PVICs that were found stretched, spindle shaped and oriented along the nanofibers of outer layers (circumferential and radial layers) of constructs, scale: 30 μm . (c–d) In confocal images, vimentin stained PVICs in fibrosa and ventricularis layers in PAV leaflets were found aligned and stretched, scale: 30 μm . Instead of SEM images, immunostained images of native layers were useful to detect cells within numerous collagen fibrils. (e–h) SEM images of collagen fibrils in (e) circumferential and (f) radial layers of TCs and in (g) fibrosa and (h) ventricularis layers of PAV leaflets. They all were found aligned in their respective layers, scale: 130 nm. (i–l) Masson's trichrome stained PVICs and collagen fibrils in (i) circumferential and (j) radial layers of TCs and in (k) fibrosa and (l) ventricularis layers of PAV leaflets. Red-stained cells (cytoplasm) (i–j) against blue-stained collagen fibrils brought violet color. The density of VICs in PAV leaflets was much less compared to that in TCs and so, PVICs in stained collagen fibrils (k–l) were less visible. The PVICs and collagen fibrils were aligned in respective layers, Scale: 155 μm . Two-headed white arrows show their respective nanofiber orientations

Figure 9.

Transmission electron microscopy images of TCs at cross-section. (a) Circumferential (x), random (y) and radial (z) layers of a TC separated by white lines were comparable to fibrosa, spongiosa and ventricularis layers of PAV leaflet, respectively, Scale: 50 μm . (b) Portion of the layer x at high magnification. Cells were greatly aligned and elongated (longitudinally) in the circumferentially oriented layer of the construct, Scale: 10 μm . Two-headed white arrow show the nanofiber orientation in the layer. (c) Portion of the layer y at high magnification. Cells had no particular orientation and shape in randomly oriented layer of the construct, Scale: 10 μm . (d) Portion of the layer z at high magnification. Cell images had specific circular or elliptical shape which confirms that the cells were in the radially oriented (perpendicular to the paper) layer of the construct, Scale: 10 μm

Figure 10.

Assesment of collagen formation in growing TCs in comparison with those in PAV leaflets through TEM imaging. (a) Cross-sectional images of a developing TC (on a copper grid) showed the formation of collagen fibrils in circumferential (x), random (y) and radial (z) layers (partitioned by white lines), respectively, scale:35 μm . (b) Cross-sectional images of a PAV leaflet (on a copper grid) showing the presence of collagen fibrils in fibrosa (x), spongiosa (y) and ventricularis layers (partitioned by white lines), respectively, scale: 40 μm . (c-d) Radially oriented layer (c, magnified image of small rectangle in z layer of figure a) of

the TC showed the presence of collagen fibrils (black arrow) as observed in the ventricularis layer (d, magnified image of small rectangle in z layer of figure b) of the PAV leaflet, scale: 240 nm. (e-f) Randomly oriented layer (e, magnified image of small rectangle in y layer of figure a) of the TC showed the presence of collagen fibrils (black arrows) as observed in the spongiosa layer (f, magnified image of small rectangle in y layer of figure b) of the PAV leaflet, scale: 240 nm. (g-h) Circumferentially oriented layer (g, magnified image of small rectangle in x layer of figure a) of the TC showed the presence of highly aligned collagen fibrils (black arrows) as observed in the fibrosa layer (h, magnified image of small rectangle in x layer of figure b) of the PAV leaflet, scale: 240 nm. Two-headed white arrow shows the nanofiber orientation in the layer

Figure 11.

Gene expression and immunostaining analysis of PVICs in TCs and PAV leaflets. (a) α -SMA, vimentin and COL1A1 expression of VICs in TCs and PAV leaflets were compared. Expression of α -SMA of PVICs in TCs was almost two and half times of that in PAV leaflets. Vimentin and COL1A1 expression were almost similar in both the TC and PAV leaflet. * denotes statistical significance $P < 0.05$ between the indicated pairs. The results shown are mean values $\pm$ SEM ($n = 3$). (b) α -SMA (red) and vimentin (green) staining corroborated the gene expression of PVICs in TCs and PAV leaflets. Vimentin staining was

observed in both TCs and leaflets showed. While α -SMA staining in TCs, it was not observed in leaflets. Circumferential layer of TCs had less positive α -SMA staining compared to their random and radial layers. Stained PVICs showed their alignments in the respective layers of TCs. Nuclei were stained blue, scales: 45 μ m. Two-headed white arrows show nanofiber orientations in respective layers. (c) Quantified α -SMA signal of PVICs on three layers of PCL TN substrates. Data refer to mean value $\pm$ standard deviation (by Student's t-test, $n = 7$, two numbers bracketed were statistically significantly different

Table 1

Tensile properties of various substrates and trilayered constructs.

	Tensile stiffness	Yield stress	Yield strain
TN substrate	0.89 ± 0.18 MPa	0.39 ± 0.10 MPa	$65 \pm 14\%$
Circumferential layer	0.33 ± 0.19 MPa	0.14 ± 0.06 MPa	$62 \pm 20\%$
Random layer	0.89 ± 0.21 MPa	0.27 ± 0.05 MPa	$78 \pm 16\%$
Radial layer	1.13 ± 0.11 MPa	0.34 ± 0.03 MPa	$49 \pm 11\%$
Trilayered construct	2.50 ± 0.67 MPa	0.86 ± 0.22 MPa	$60 \pm 18\%$