

Cellular Response to Cyclic Compression of Tissue Engineered Intervertebral Disk Constructs Composed of Electrospun Polycaprolactone

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There is lack of investigation capturing the complex mechanical interaction of tissue-engineered intervertebral disk (IVD) constructs in physiologically relevant environmental conditions. In this study, mechanical characterization of anisotropic electrospinning (ES) substrates made of polycaprolactone (PCL) was carried out in wet and dry conditions and viability of human bone marrow derived mesenchymal stem cells (hMSCs) seeded within double layers of ES PCL were also studied. Cyclic compression of IVD-like constructs composed of an agarose core confined by ES PCL double layers was implemented using a bioreactor and the cellular response to the mechanical stimulation was evaluated. Tensile tests showed decrease of elastic modulus of ES PCL as the angle of stretching increased, and at 60 deg stretching angle in wet, the maximum ultimate tensile strength (UTS) was observed. Based on the configuration of IVD-like constructs, the calculated circumferential stress experienced by the ES PCL double layers was 40 times of the vertical compressive stress. Confined compression of IVD-like constructs at 5% and 10% displacement dramatically reduced cell viability, particularly at 10%, although cell presence in small and isolated area can still be observed after mechanical conditioning. Hence, material mechanical properties of tissue-engineered scaffolds, composed of fibril structure of polymer with low melting point, are affected by the testing condition. Circumferential stress induced by axial compressive stimulation, conveyed to the ES PCL double layer wrapped around an agarose core, can affect the viability of cells seeded at the interface, depending on the mechanical configuration and magnitude of the load. [DOI: 10.1115/1.4039307]

Keywords: electrospinning, tissue engineering, confined compression, annulus fibrosus, intervertebral disk

Introduction

Treatment strategy involving scaffold implants and cell delivery has been proposed to aid restoration of the biomechanics of intervertebral disk (IVD) via contributing to the repair of degenerated IVD [1,2]. Replication of the annulus fibrosus (AF) structure is particularly challenging due to its multilayered anisotropic organization with fibers oriented at alternating angles. Mechanical properties similar to the native tissue are fundamental for the success of AF scaffold as the containment of nucleus pulposus by a stack of layers of AF plays a fundamental role [3].

Scaffolds with fibril anisotropic structures have been investigated for application as scaffolds in tissue engineering, in particular electrospun (ES) matrices. The mechanical properties of ES matrices depend on fiber diameter, density, and relative arrangement, apart from the polymer material property itself. All these parameters also influence the cellular response when scaffolds are used for AF tissue engineering. Modifications of fiber diameter contribute to matrix bulk mechanical properties and, simultaneously, to fiber packing density, which, in turn, affect the number of sites and the porous structure available for cell attachment and cell infiltration [4]. Mechanical characterization of several potential electrospun scaffolds for tissue engineering applications has

been performed [5–8]. Similar polycaprolactone (PCL) constructs have been tested in various orientations [9], in stacks [10], wrapped around agarose gels and mechanically tested [11], and implanted into animal models [12,13]. However, there is lack of investigation capturing the complex mechanical interaction of tissue engineered ES scaffolds in some physiologically relevant environmental conditions.

In the present work, the mechanical compliance of AF constructs, fabricated from angle-ply laminates of ES PCL matrix, were tested under uniaxial cyclic confined compression to simulate the loading condition of the IVD. We demonstrated that loading conditions applied to the constructs can affect the viability of human bone marrow derived mesenchymal stem cells (hMSCs) embedded in the structures, representing AF in an IVD-like configuration. Mechanical characterization of anisotropic ES substrates made of PCL was also carried out and viability of human bone marrow derived mesenchymal hMSCs seeded within the double layers of ES PCL was further studied. Cyclic compression of IVD-like constructs composed of an agarose core confined by ES PCL double layers was implemented using a bioreactor and the cellular response to the mechanical stimulation was evaluated.

Materials and Methods

Matrix Fabrication and Morphology Analysis. Polycaprolactone was electrospun according to the procedure described previously [14]. Briefly, PCL was dissolved in 1:1 mixtures of

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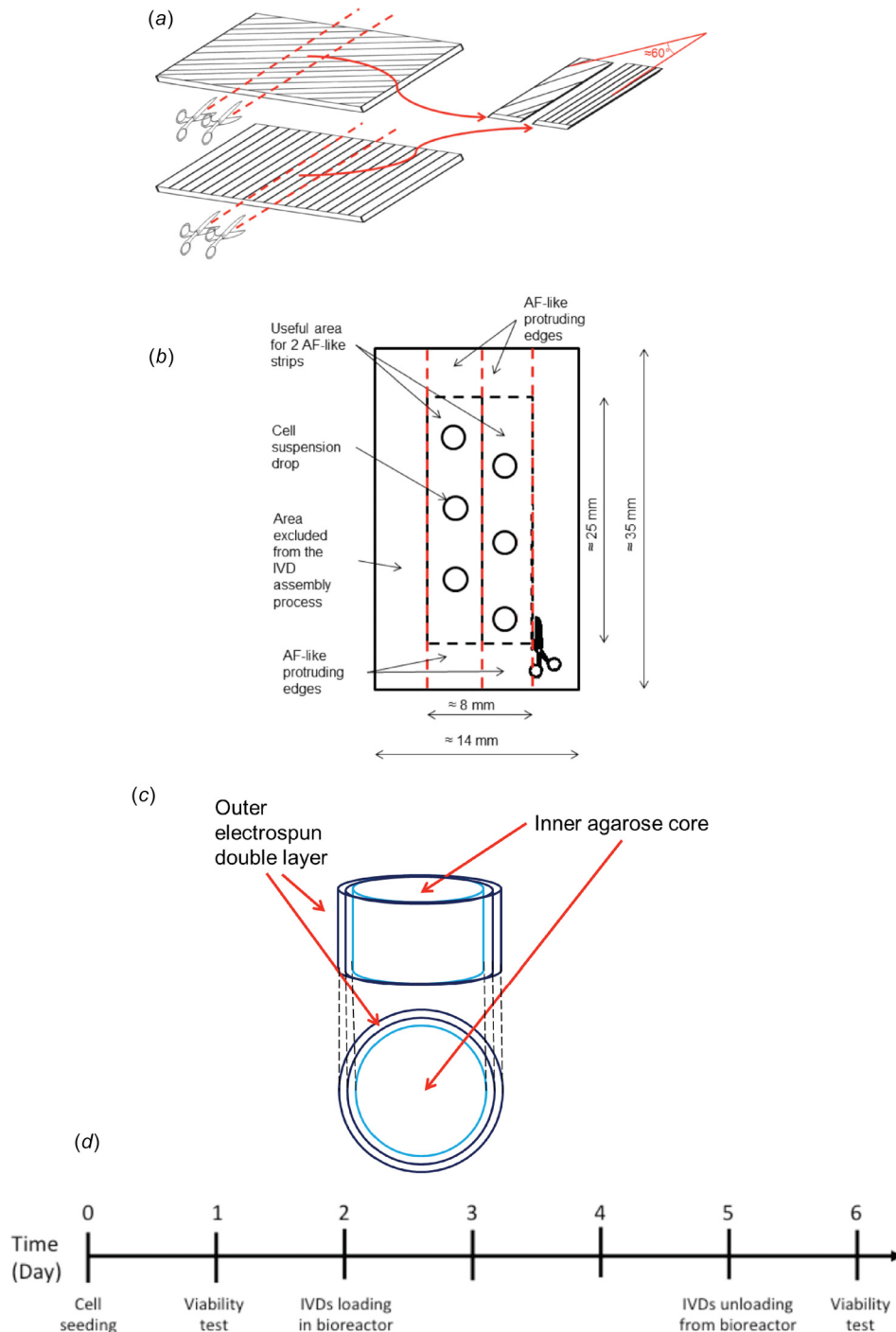


Fig. 1 Schematics representing the angle-ply configuration of double layers and the preparation for fluorescence microscopy analysis (a). Cell seeding strategy of the ES PCL matrix prior to overlaying with the top layer for the IVD-like construct preparation (b) and the structure of the final construct (c). Timeline of cell seeding, loading on and retrieving from bioreactor for test (d).

Table 1 Bioreactor mechanical stimulation parameters

	Test A	Test B
Test duration		Three days
Stimulation type		Sinusoidal compression
Stimulation pattern		Eight hours/day dynamic compression + 12 h/day static compression
Frequency		1 Hz
Dynamic displacement	10%	5%
Static compression	5%	2.5%
Hydrostatic pressure		Atmospheric

tetrahydrofuran (Carlo Erba, Milan, Italy) or chloroform (VWR, Milan, Italy) and dimethylformamide (Sigma-Aldrich, Milan, Italy) in 10–17 wt % concentration range. Voltage of 10 kV and 20 kV was applied (SL150, Spellman) between the metal nozzle (1 mm inner diameter), which is able to move parallel to the collector, and the drum collector (80 mm diameter). A syringe pump (Nexus 6000, Chemix) was used to provide a flow rate in the range of 2–3 ml/h and aligned fibers were fabricated by setting the

drum collector velocity at 3000 rpm. Surface morphology of ES PCL matrices was observed using a scanning electron microscope (SEM, Sigma FE, Zeiss). Scanning electron microscope images of fiber were analyzed by means of opportune software (Nonwovens Anisotropy V1, MATLAB) to detect fiber preferential orientation [15]. Matrices thickness was measured three times in three different locations per matrix using an optical profilometer (Talysurf CLI 2000, Taylor Hobson).

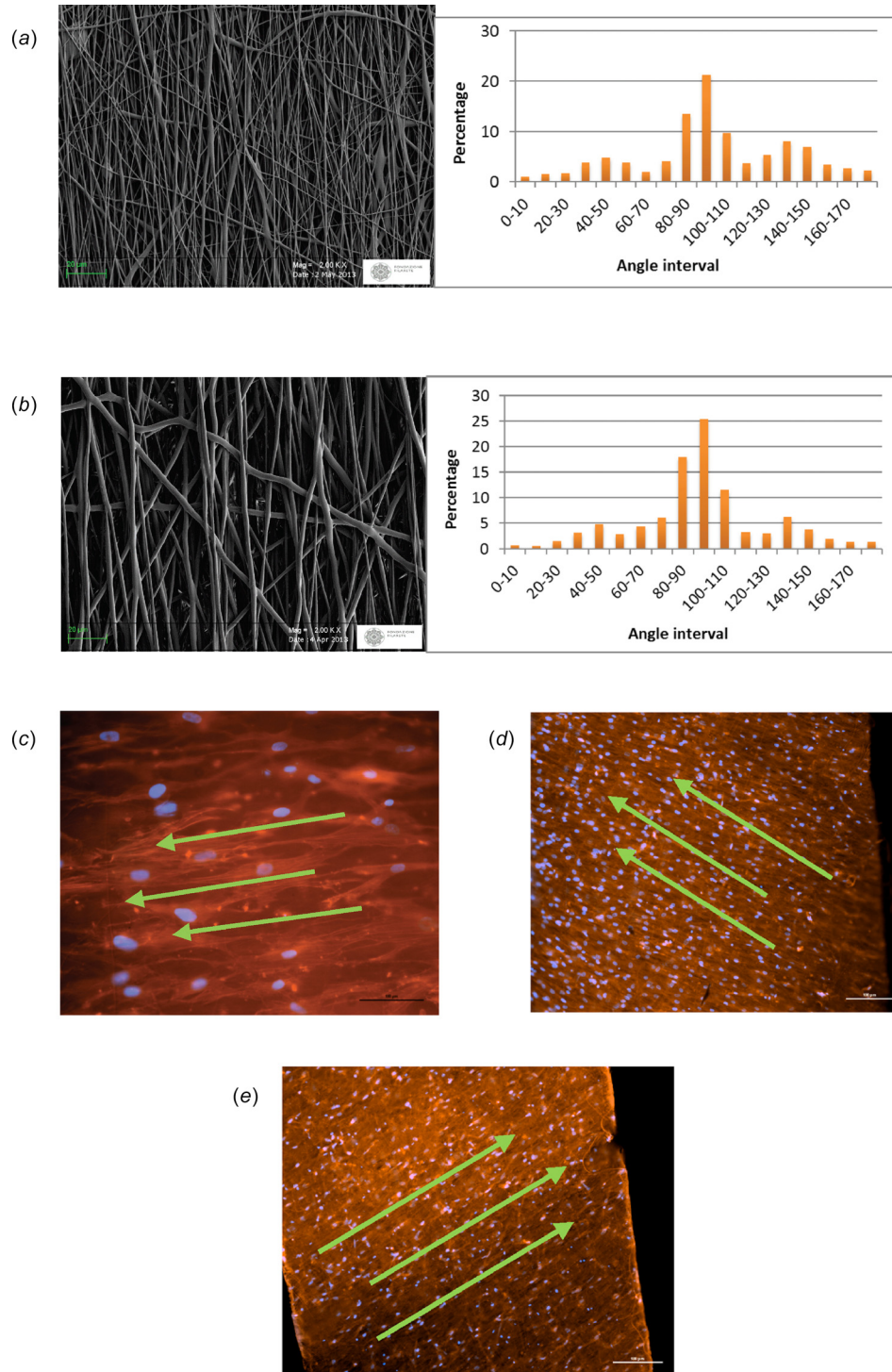


Fig. 2 (a) and (b) Representative SEM images of aligned fibers with 0.46 ± 0.14 (a) and $1.72 \pm 0.50 \mu\text{m}$, (b) fiber diameter. Histograms represent the angle distribution of ES PCL fibers. Scale bar $20 \mu\text{m}$, (c)–(e) hMSCs attached to aligned fibers arranged into monolayer (c) and double layer, bottom (d) and top (e) layer. Cell nuclei are stained blue and actin filaments red. Scale bar $100 \mu\text{m}$.

Tensile Test in Dry and Wet Conditions. Mechanical properties of aligned PCL fibers were evaluated by tensile testing at room temperature in air (dry conditions) and at 37 °C in phosphate buffered saline. Rectangular specimens (10 × 40 mm²) were cut from the matrix with the longest axis of the specimens and the main fiber direction arranged at 0 deg, 30 deg, 60 deg, and 90 deg angles, respectively. Instron 3366 tensile testing machine was set to preload the sample at 0.5 N, followed by stretching to failure at a stretching rate of 10 mm/min. The elastic modulus was calculated according to the ratio between the stress and the strain in the linear region of low strain following a computational fitting.

Preparation of Electrospinning Polycaprolactone Multiple Layer Constructs Seeded With Cells. hMSCs (Lonza) were cultured in low glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% antibiotic and antimycotic, 1% L-Glutamin, and 1% nonessential amino acids. Cells were trypsinized and seeded onto ES PCL matrices with aligned fibers at a density of 5 × 10⁴ cells/sample. To prepare double layer constructs, after 24 h of culture each monolayer was overlaid with another fresh layer of ES PCL matrix. Double layers with angle-ply laminate configuration were realized by maintaining an angle of 60 deg between the main fiber direction of the top and bottom layers (see Fig. 1(a)). Viability of hMSCs cultured on ES PCL double-layer matrix with thickness of 77.97 ± 10.28 and 155.94 ± 20.56 μm and fiber diameter 0.46 ± 0.14 and 1.72 ± 0.50 μm was investigated, respectively, using Alamar Blue (AB) (Life Technologies) assay. At the end of culture, samples were thoroughly rinsed in phosphate buffered saline and fixed in formalin (Sigma) twice for 1 h. Phalloidin Alexa Fluor-555 (Life Technologies) and Hoechst 33342 (Life Technologies) dyes were selected to stain cells actin filaments and nuclei, respectively. To preserve the original angle-ply configuration of double layers, a rectangular narrow strip was cut out and the two layers were then separated and placed parallel to each other with the inner side facing the objectives of the microscope (Fig. 1(a)).

Intervertebral Disk-Like Construct Assembly and Mechanical Conditioning. Intervertebral disk-like constructs were prepared by wrapping around an agarose core with ES PCL double layers seeded with hMSCs. A 2% w/v agarose solution was used to prepare agarose cores 8 mm in diameter and 3.7 mm in height. Two ES PCL double-layer strips with size of 8 mm in width, 35 mm in length, and 173.88 μm thickness (86.94 μm for individual layer) were cut out from the middle portion of each double layer and applied as rims to seal around the agarose disks (Figs. 1(b) and 1(c)) (3.7 mm height and 8 mm diameter) with commercial waterproof polyurethane resin (Wilkinson) (the glue had been pretested for biocompatibility). Intervertebral disk-like constructs were loaded onto and cultured in the compressive bioreactor (Bose ElectroForce® 5900 BioDynamic® Test Instrument) and the timeline of culture is shown in Fig. 1(d). Sinusoidal mechanical conditioning at 1 Hz with 10% or 5% vertical displacement was conducted for 8 h, followed by static compression of 5% or 2.5% displacement (see Table 1). As a control, cell seeded IVD-like constructs were cultured in static conditions in the same incubator as the bioreactor.

Hoop Stress Mathematical Analysis. Hoop stress was calculated based on the assumption that the compressive stress was totally transmitted to the wall as internal pressure and the thin wall assumption of nonwoven fabric ES PCL. The internal pressure (P) is generated by the compressive load applied to the agarose disk according to

$$P = F/A_0$$

where F is the compressive load applied and derived from the load and displacement curve of agarose core of IVD-like constructs under confined compression obtained using WinTest®

control software of the bioreactor and A_0 is the initial cross-sectional area of the agarose disk. In order to calculate the hoop stress (σ_H) applied on the ES PCL, thin wall cylinder assumption will be made according to

$$\sigma_H = P \cdot r/t$$

where r is the radius of the constructs and t is the thickness of the ES PCL layer in cylinder shape.

Statistical Analysis. Results are presented as average of triplicates ± standard deviation. T-Student and two-way analysis of variance statistical tests were performed using SPSS® (IBM).

Results

Morphology and Mechanical Properties of Electrospinning Polycaprolactone Matrix With Aligned Fibers. Matrix with two types of surface morphology is presented in Fig. 2, one with small fiber diameter 0.46 ± 0.14 μm and higher fiber density (Fig. 2(a)), and the other with large fiber diameter 1.72 ± 0.50 μm and low fiber density (Fig. 2(b)). The fiber preferential orientation is shown as the histogram. Most of the fibers are distributed at the

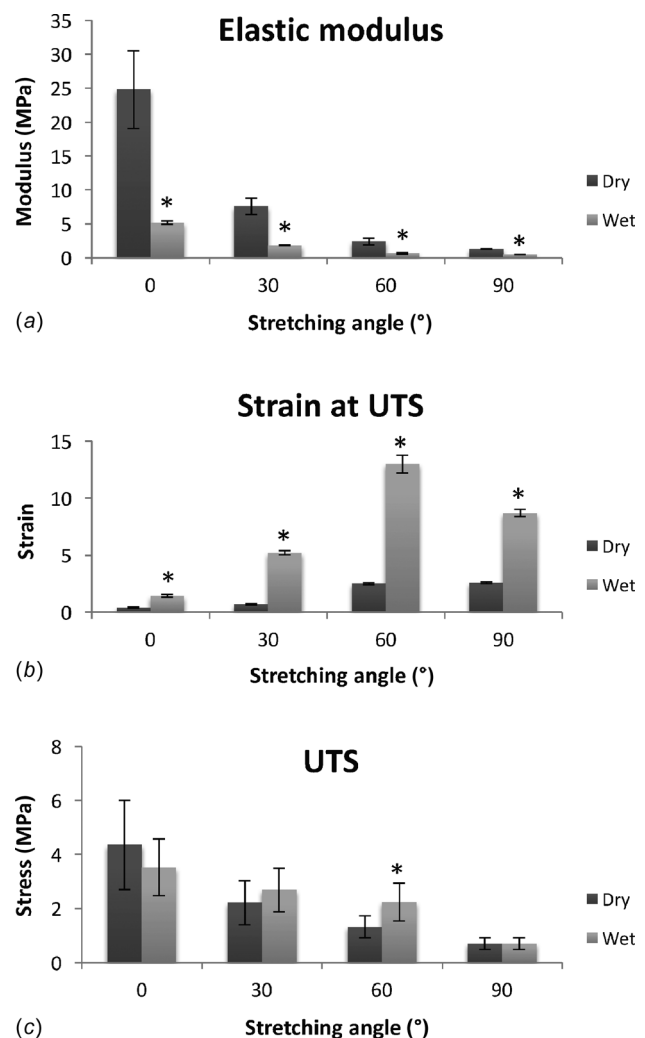


Fig. 3 Mechanical properties of aligned ES PCL fibers stretched at different angles in dry and wet conditions. (a) Elastic modulus, (b) strain at ultimate tensile strength, and (c) ultimate tensile strength. Note: asterisks indicate significant difference ($p < 0.05$) between dry and wet conditions within the same stretching angle.

angle intervals of 80–110 deg with a peak at 90 deg, which correspond to the electrospinner drum collector rotation direction, together with some distribution in the subdomains of 40–50 deg and 140–150 deg. The mechanical properties of matrices of aligned fibers (see Fig. 3) were significantly affected by both the stretching angles and the testing conditions. There is a clear trend of decrease of moduli when the stretching angle was changed from 0 deg to 90 deg and when samples were tested in wet condition (Fig. 3(a)). At 0 deg in dry conditions, the modulus was the highest (24.8 ± 5.72 MPa), approximately five times higher than those in wet conditions. However, with the increase of stretching angle, the difference between dry and wet moduli was less dramatic, but still statistically significant. The combinational effects of stretching angle and environmental conditions also affect the strain measured at ultimate tensile strength (UTS) (Fig. 3(b)). The highest strain (13.00 ± 0.78) was observed in wet conditions at 60 deg stretching angle, followed by 90 deg (8.69 ± 0.32) and 30 deg (5.21 ± 0.17), respectively. At 0 deg, although the difference between the wet and the dry is not dramatic, still ES PCL matrices in wet have higher strain than those in dry conditions (1.42 ± 0.12 against 0.38 ± 0.07). Ultimate tensile strength also decreased with the increase of stretching angle from 0 deg to 90 deg (Fig. 3(c)). The difference of UTS between those tested in dry and wet conditions was significant only when samples were stretched at 60 deg.

Human Bone Marrow Derived Mesenchymal Stem Cells Cultured on Double Layers of Electrospinning Polycaprolactone. Figures 2(c)–2(e) show an almost homogenous distribution of attached and elongated hMSCs covering the surface of ES PCL monolayer. Elongation of cell bodies is shown clearly in Fig. 2(c) with actin filaments and cell nucleus staining red and blue, respectively. Large populations of cells can be seen on the overlaid top layers (see Fig.

2(e)), confirming the capability of cells to migrate across the planes of double layers. Moreover, it seems the migrated cells elongated in the same direction as the aligned fibers of the overlaid layers, which was arranged in a manner of 60 deg angle-ply between the bottom layer (Fig. 2(d)) and the top overlaid layer (Fig. 2(e)).

The effect of ES PCL, with varied matrix thickness (thin: 77.97 ± 10.28 and thick: 155.94 ± 20.56 μm , fiber diameter 0.70 ± 0.38 μm for both) and fiber diameters (small: 0.46 ± 0.14 and large: 1.72 ± 0.50 μm), on hMSCs viability cultured at the interface of double layers was also studied and shown in Figs. 4(a) and 4(b). Generally, cells seeded on double layers showed trend of growth similar to the control during the 21 days of culture. Although a lag phase was presented in the first week for the double layers, a log phase growth was observed from day 7 onward. Viability of cells seeded between thicker double layers was lower by day 7 than those on the thinner one and the control, but by 14 days there was no dramatic difference compared to the control. After 21 days of culture, cell viability was found higher on double layers with lower thickness ($p < 0.05$, Fig. 4(a)). By day 21, cells cultured on matrices showed higher viability, particularly those on small fibers (Fig. 4(b)).

Mechanical Conditioning of Intervertebral Disk-Like Constructs. Figure 5(a) shows the final IVD-like construct prepared by wrapping an angle-ply double-layer ES PCL seeded with hMSCs around a 8 mm diameter agarose gel. The configuration of the IVD-like constructs allowed execution of confined compression to the agarose core as evidenced by the continuous increase of stress with the progress of vertical strain (see Fig. 5(b)). In Fig. 5(b), the difference between the stress–strain curves measured on agarose only and agarose surrounded by nanofibers is shown. Not only the presence of nanofibers increases the slope (i.e., higher

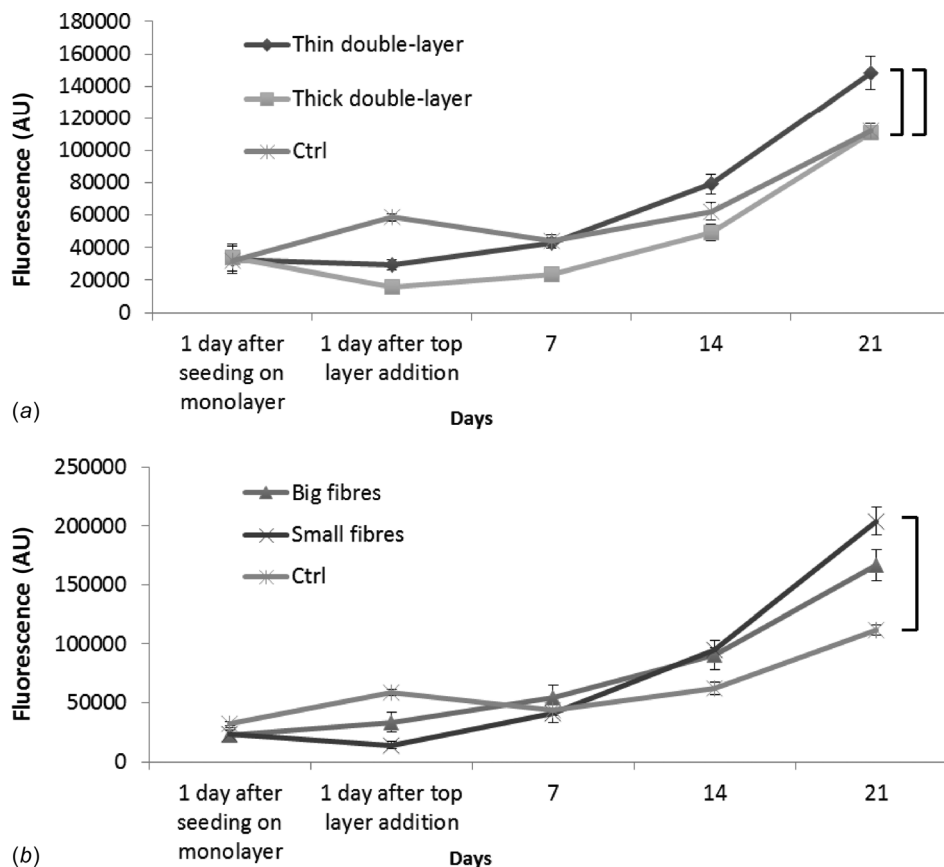
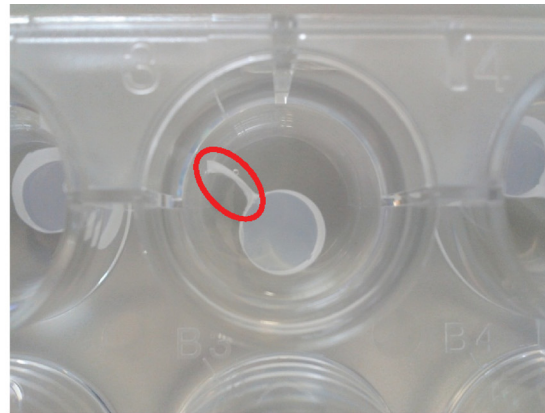
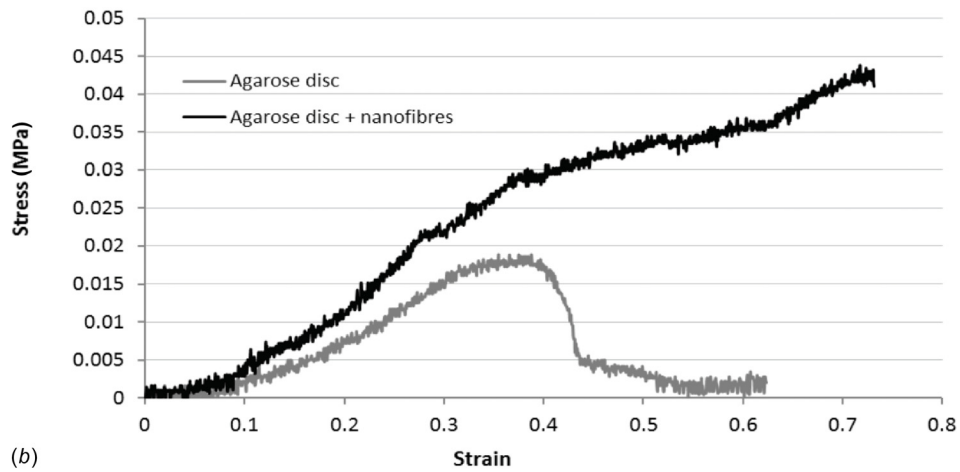


Fig. 4 (a) Viability of cells seeded on double layers of different fiber diameters and (b) viability of cells seeded on double layers characterized by different thicknesses. Black bars on the right-hand side indicate significant difference ($p < 0.05$).

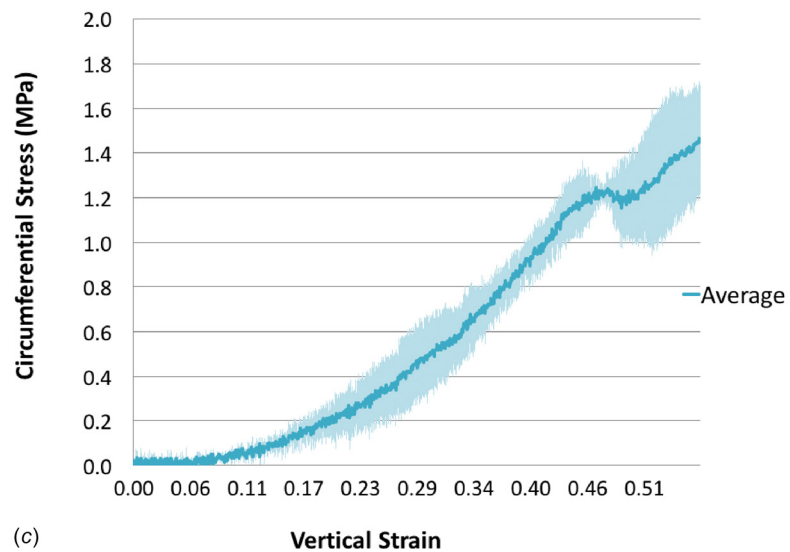


(a)

Representative S-S curve for agarose disc and agarose disc surrounded by nanofibres

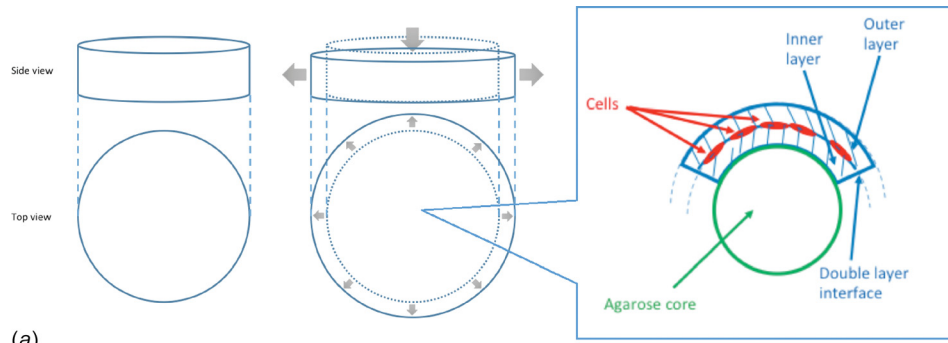


(b)

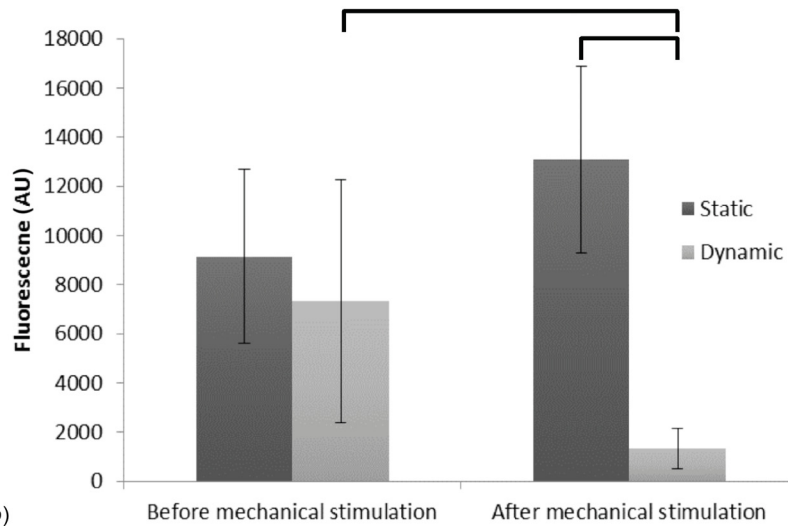


(c)

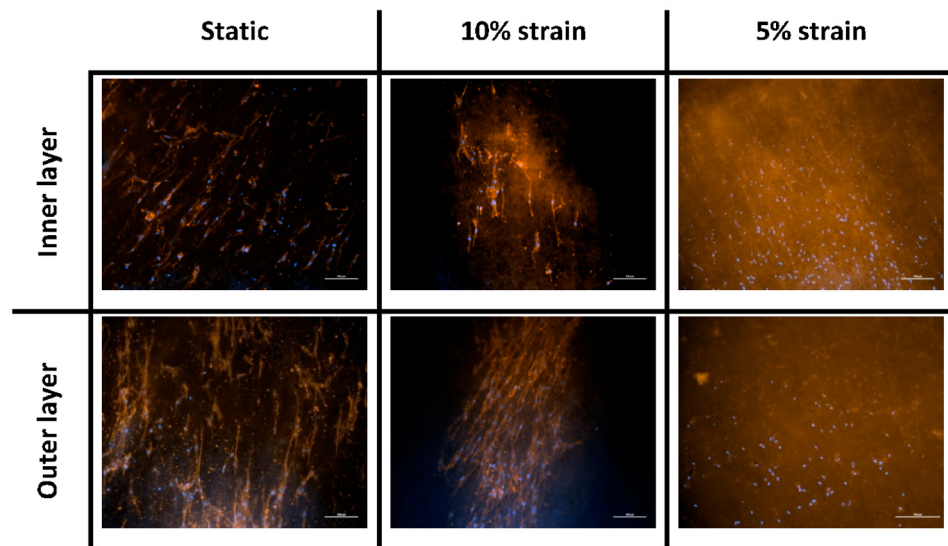
Fig. 5 (a) Agarose disc surrounded by an AF-like double-layer strip. The protruding edges labeled with red circle were used for handling the specimen during the sealant process and for loading onto the bioreactor. (b) Representative S-S curves of agarose disc and agarose disc surrounded by ES PCL layers. (c) Calculated circumferential stress experienced by the double-layer ES PCL layers from the average of three samples.



(a)



(b)



(c)

Fig. 6 (a) Schematic diagram showing the translation of compressive stress to circumferential stress experienced by the double layers of ES PCL with cells presented at the interface. (b) Viability of 5% mechanically stimulated IVD-like constructs. The assay was performed on both static and stimulated samples, at the same time points, before and after applying mechanical stimulation. Bars indicate $p < 0.05$ significance. (c) Representative images acquired at the end of mechanical stimulation experiments. Inner layer is the one in contact with the agarose gel and cells facing outward; the outer layer wraps both the inner layer and the agarose core with cells facing inward. Scale bar 100 μm .

stiffness) but also delays the breakdown of the construct, which occurs at lower strain for agarose only samples. The chord moduli measured were 96.71 ± 17.81 kPa and 145.64 ± 35.37 kPa for agarose disks and IVD-like constructs, respectively. The circumferential stress calculated accordingly from an average of three samples was 40 times of vertical compressive stress (see Fig. 5(c)).

Agarose hydrogel core of IVD-like cellularized constructs was subject to 3 days sinusoidal compression at 5% or 10% magnitude of vertical displacement (see Fig. 6(a)). Viability results of samples subject to 5% displacement are shown in Fig. 6(b). With 5% vertical displacement, viability of cells was significantly lower compared to those in the static culture and those before mechanical stimulation. When vertical displacement was further increased to 10%, the viability of cells was not detectable. The reduction on cell viability after 6 days of mechanical conditioning is dramatic, while no significant difference between day 0 and day 6 samples was noticed in static culture.

At the end of the mechanical conditioning, samples were retrieved and the interface of the double layers, where cells were preloaded, was observed and fluorescent images of cell nuclei and actin are shown in Fig. 6(c). Samples in static culture revealed a fairly even cell distribution on both inner and outer layers of samples. Samples exposed to 10% displacement had much less cells and it only presented in small isolated areas, while certain areas were totally depleted of cells (Fig. 6(c)). Specimen subject to 5% dynamic displacement showed similar distribution of cells to those exposed to 10% displacement, but the area lack of cell presence was considerably smaller. Despite the patchy cell distribution, there was no obvious difference observed between the inner and outer layers of mechanically conditioned samples and also of those in static culture.

Discussion

In the view of adopting electrospinning matrix for tissue engineering purpose, it is essential that mechanical properties of matrices were characterized under physiologically relevant conditions, in particular considering the anisotropic structure of AF. In wet conditions with the presence of water at 37°C , all the moduli decreased due to the low melting and glass transition point of PCL. At 60 deg stretching angle, fiber straightening and friction occurred with fibers interaction allow ES PCL to fail at higher strain. In addition, the presence of fibers with orientation distribution in the subdomains at 40–50 deg and 140–150 deg could also be a potential internal factor contributing to the outstanding mechanical performance of the ES PCL matrices in wet at 60 deg. The higher ES PCL UTS and strain at UTS observed at 60 deg angle of stretching in the presence of water could be extended further to the varied mechanical behavior of AF at anterior/posterior and superficial/deep tissue regions [16], in analogy to the contribution of water molecular content in glycosaminoglycans to the structure-function relationship in the native AF tissue. Glycosaminoglycans play an important role on mechanics of annulus laminates via enabling the fiber interaction during loading [17], similar to the ES PCL fiber interaction under tension in the wet condition of the current study.

Within IVD-like constructs, the confinement exerted by the ES PCL layer allowed the translation of vertical compressive stress to the ES PCL double layers as radial and circumferential stress (Fig. 7(a)). Similar concept of re-establishment of the mechanical interaction via simulation of the confined compression of IVD had been reported by Lazebnik et al. [18]. It has been demonstrated that angle-ply laminate structure would allow dissipation of the energy of biaxial tension via fiber stretching, and hence, could prevent shearing within each lamella [19]. When correlating with the measured elastic modulus of aligned fibers in wet at stretching angle 60 deg, the circumferential stress at 0.05 and 0.1 vertical true strain could lead to 0.087 and 0.23 circumferential strain of the ES PCL layer (see Fig. 7(a)), which should be still under the strain of yield $30\% \pm 11\%$ according to those published by Baker et al. [20]. Therefore, it is possible that the low viability observed

could be due to the destabilization of focal adhesion sites of cells in directional response to the extensively stretched fibers. However, the patchy cell distribution on IVD-like constructs also indicated nonuniform interaction between the laminates of the double layers. It is anticipated that when ES PCL double layers with ± 60 deg angle-ply configuration are subject to tension from the circumferential stress, the angle between adjacent layers decreases proportionally to the displacement as the consequence of fiber in-plane rotation into the direction of stress (Fig. 7(b)). Such fiber interaction within laminate equivalent to linear and shear strain between collagen fiber bundles of single AF laminate also supports that fibers interaction should be the main component of strain within a lamella as inferred and proposed by Vergari et al. [21]. Future study should focus on understanding of the complex mechanical behaviors of ES fibers and network formed at microscopic level, correlating to cell adhesion and cell response to mechanical stress.

The reduced cell viability and density, particularly in the region of outer AF, had been reported by Paul et al. after exposure of IVD to high dynamic loading [22]. High magnitude load is known to trigger IVD cells catabolic processes [23] and apoptosis [24] and decreased cell viability [25]. It is understood that the outer layers of the AF are more likely to be exposed to much greater strain due to their greater distance from the center of the rotation of the disk, hence, have higher mechanical strength to resist delamination [26]. One of the limitations of the current study is that only small magnitude of mechanical interaction and corresponding circumferential stress can be achieved due to the limitation of the diameter of the IVD-like constructs (8 mm). In addition, the compressive modulus of the IVD-like construct of the present study is inferior to that of the human IVD [27], but corroborate 0.116 ± 0.127 MPa aggregate modulus of confined

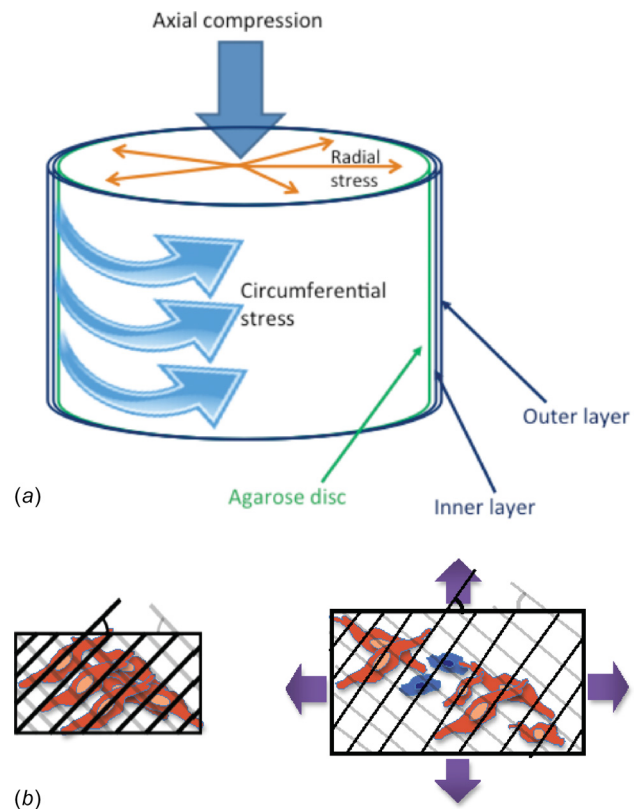


Fig. 7 (a) Schematic diagram showing the translation of compressive stress by the configuration of the IVD-like constructs and (b) impaired cell attachment and viability as a consequence of fiber stretching and rotation in response to biaxial tensile stress (cells labeled blue represent those cells with compromised viability)

compressed AF reported [3,28]. Still the experimental model of circumferential stress within an angle-ply laminated structure presented here can aid future experimental approach to gain understanding of the mechanobiological mechanisms of delamination between annular lamellae [26,29].

Conclusions

Circumferential stress induced by axial compressive stimulation can be effectively conveyed to the hMSCs seeded between the outer ES PCL double layer wrapped around an agarose core. Such mechanical stimulation can affect the viability of cells seeded at the interface, depending on the mechanical configuration and magnitude of the load.

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