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Annulus fibrosus tissue engineering using lamellar silk scaffolds

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Abstract

Degeneration of the intervertebral disc (IVD) represents a significant muscular skeletal disease. Recently, scaffolds composed of synthetic, natural and hybrid biomaterials have been investigated as options to restore the IVD; however, they lack the hallmark lamellar morphological features of annulus fibrosus (AF) tissue. The goal of regenerating disc is to achieve anatomic morphology as well as restoration of mechanical and biological function. In this study, two types of scaffold morphologies formed from silk fibroin were investigated towards the goal of AF tissue restoration. The first design mimics the lamellar features of the IVD that is associated with the AF region. The second is a porous spongy scaffold that serves as a control. Toroidal scaffolds were formed from the lamellar and porous silk material systems to generate structures with an outer diameter of 8 mm, inner diameter of 3.5 mm and a height of 3 mm. The inter-lamellar spacing in the lamellar scaffold was 150–250 μm and the average pore sizes in the porous scaffolds were 100–250 μm . The scaffolds were seeded with porcine AF cells and, after growth over defined time frames *in vitro*, histology, biochemical assays, mechanical testing and gene expression indicated that the lamellar scaffold generated results that were more favorable in terms of ECM expression and tissue function than the porous scaffold for AF tissue. Further, the seeded porcine AF cells supported the native shape of AF tissue in the lamellar silk scaffolds. The lamellar silk scaffolds were effective in the formation of AF-like tissue *in vitro*.

Keywords

Lamellar; Silk; Annulus fibrosus (AF); Intervertebral disc (IVD)

1. Introduction

Low Back Pain (LBP) is generally associated with degeneration of the intervertebral disc (IVD) (Anderson 1986). IVD degeneration is characterized, in its late stages, by progressive microstructural derangement of the annulus fibrosus (AF) extracellular matrix. Damage to the AF is attributed to mechanical causes, biological remodeling, loss of nutrition, and accumulation of cellular waste products (Iatridis *et al.*, 2005). Current treatment modalities

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involve conservative management (mediation and physical therapy) or surgical intervention (spine fusion, total disc replacement, or nucleus pulposus (NP) replacement). However, the focus of these methods is to gain symptomatic relief by removal of disc tissue, without specifically identifying the underlying biological problem. In addition, these surgical procedures have limited success rates and they are not applicable to all patients (Hegewald *et al.*, 2008). This issue has led to interest in biological repair of damaged disc tissues using tissue engineering methods. IVD tissue engineering presents the opportunity to restore the functionality of the IVD by repairing or replacing the degenerated tissue (O'Halloran and Pandit 2007).

The IVD is a complex structure that can be separated macroscopically into at least two anatomic zones: the NP, representing a centrally located gelatinous homogeneous mass, and the AF, consisting of concentrically organized layers of collagen fibrils which surround the NP (Kluba *et al.*, 2005). Both tissues contain an abundant matrix of negatively charged proteoglycans entangled with collagen fibers. In particular, the AF consists of an extracellular matrix (ECM) composed of both type I and type II collagen oriented in a lamellar structure with a predominance of type I collagen (Richardson *et al.*, 2006, Wan *et al.*, 2008). The fundamental tension-bearing elements are bundles of type I collagen fibrils, which are arranged obliquely to the axial plane of the disc in discontinuous, approximately concentric lamellae around the nucleus (Marchand and Ahmed 1990). Proteoglycans, principally aggrecan, represent the second greatest constituent of the disc in terms of dry weight after collagen constituting 5–8% of the outer annulus and 11–20% of the inner annulus (Feng *et al.*, 2006).

For successful cell-based tissue engineering, cells should interact with an appropriate scaffolding material that closely mimics the structural, biological, and mechanical functions of native ECM (Venugopal and Ramakrishna 2005). Therefore the goal of regenerating the disc tissue should not only be to achieve restoration of anatomic morphology, but also to restore function. Engineering a functional replacement for the AF of the IVD is contingent upon recapitulation of the AF structure, composition, and mechanical properties. While a wide variety of biomaterials have been studied for use in articular cartilage tissue engineering, fewer studies have been conducted for disc tissue engineering in spite of the importance of this tissue (Frenkel and Di Cesare 2004, Raghunath *et al.*, 2007). AF tissue engineering approaches have utilized scaffolds of collagen (Sato *et al.*, 2003), agarose (Gruber *et al.*, 2006), collagen-GAG (Rong *et al.*, 2002), alginate-chitosan (Shao and Hunter 2007), and polyglycolic acid and polylactic acid (Mizuno *et al.*, 2004). Recently, a few studies have induced AF tissue formation using lamellar scaffolds, since native AF tissue consists of a lamellar oriented structure. Shao used an alginate/chitosan scaffold to form a lamellar AF structure and showed that this scaffold supported canine AF cell growth and function (Shao and Hunter 2007). Wan *et al.* used polycaprolactane triol malate with demineralized bone matrix gelatin (BMG/PPCLM) for the inner and outer layers of the AF (Wan *et al.*, 2008). Although these studies utilized scaffolds that supported cell growth and desired phenotype, the scaffolds did not recapitulate the architecture of the AF (Chang *et al.*, 2010). *Bombyx mori* silk material was utilized in the present study due to biocompatibility, biodegradability and tough material properties, as well as its ability to be reprocessed into various material formats (Vepari and Kaplan 2007). Silk porous scaffold have already demonstrated utility for AF tissue engineering; however, the results showed that tissue growth was not uniformly distributed throughout the scaffold (Chang *et al.*, 2007). In addition, although AF tissue formation and better cell distribution was found on the porous silk scaffolds under dynamic spinner flask culture, the lamellar AF structure was not mimicked (Chang *et al.*, 2010).

The success of a cell-based, polymeric tissue engineered disc graft relies on its ability to function as a 3D support matrix mimicking the native structure in order to help with graft integration, and to support cell proliferation and production of tissue-specific ECM (Temenoff and Mikos 2000). In the present study, a simplified freeze drying technique was utilized to generate lamellar scaffolds for engineering functional units of the AF. This lamellar scaffold was compared to non-lamellar porous scaffolds as controls for AF-like tissue formation. The purpose of this study was to determine whether a lamellar scaffold system based on silk would provide improved AF tissue formation and function *in vitro*. To explore the utility of this scaffold for tissue engineering, cell growth was characterized by scanning electron microscopy (SEM), histology and immunostaining. In addition, chemical analysis (collagen and glycosaminoglycans), q-PCR and mechanical testing were used to assess outcomes.

2. Materials and Methods

2.1. Isolation & culture of annulus fibrosus cells

Porcine AF cells were kindly provided by the University of Tennessee Health Science Center (UTHSC). AF cells were isolated from porcine IVD. In brief, intervertebral discs were obtained from the lumbar disc of porcine (2–3 weeks old). The spine was sectioned between each of the lumbar discs from T10 to L5. The muscles and tendons were removed, and the column was sectioned transversally in the middle of each disc. The surrounding AF was separated from upper and lower vertebral cartilage and excised so that the surrounding ligament to which it is joined was discarded. Cells from the AF tissues were isolated by 1–2 hours digestion at 37 °C in 0.05% pronase (Boehringer Mannheim), followed by overnight digestion at 37°C in 0.2% collagenase (Worthington Biochemicals, Lakewood, NY) using modified DMEM/F12 (Gibco BRL, Grand Island, NY) medium with 5% fetal calf serum (FCS, Gibco BRL), 4.8 mM CaCl₂ and 40 mM HEPES buffer (Sigma-Aldrich). After 18 hours of shaking the completely digested specimens had released cells, which were confirmed by phase contrast microscopy. The digested samples were centrifuged at 250 *g* for 5 minutes to isolate the AF cells for counting. The cells were counted using a hemacytometer and cell numbers and viability were determined using a trypan blue exclusion test. The cells were then plated at a density of 1.5×10^5 cells/cm² and placed at 37 °C in a 5% CO₂ incubator. The DMEM/F12 culture medium, which included 10% fetal bovine serum (Gibco), 1% antibiotic-antimycotic (Gibco), 50 µg/mL ascorbic acid (Sigma, St Louis, MO) was changed every other day. The primary AF cells were passaged twice before the experiments.

2.2. Preparation of silk solution

Silk fibroin (SF) solutions were prepared according to procedures described previously (Kim *et al.*, 2005, Kim *et al.*, 2005). Briefly, 6–8% (w/v) silk fibroin solution was prepared from *Bombyx mori* silkworm cocoons. The cocoons were extracted in a 0.02 M Na₂CO₃ solution, dissolved in a 9.3 M LiBr solution and subsequently dialyzed against distilled water.

2.3. Preparation of lamellar silk scaffolds

To generate lamellar structure, no salt was added and instead a 1.5 mL 4% SF/ 0.2 % sodium alginate solution mixture was added to a silicone mold (12 mm diameter, 5 mm thick) with one side capped with parafilm. Immediately the molds were placed in a freezer at –80°C for 2 hrs. Subsequently, the scaffolds were lyophilized for 2 days and then water annealed for 6 hours to generate the insoluble state of silk by inducing beta sheet crystallinity (Jin *et al.*, 2005). The scaffolds were then submerged in water for 24 hours to remove the mixed alginate. Toroidal disk scaffolds were formed out of the lamellar structure to generate an outer diameter of 8 mm, inner diameter of 3.5 mm and a height of 2~3 mm

using disposable punches (Acuderm Inc, Fort Lauderdale, FL). Toroidal silk disks were submerged in 70% EtOH for sterile cell cultivation and were conditioned with the culture medium overnight before seeding cells. (Fig. 1a)

2.4. Preparation of porous silk scaffolds

2 mL aliquots of a 4% silk fibroin solution with 50 mM carbodiimide (EDAC) and 20 mM N-hydroxysuccinimide (NHS) in Teflon cylinder containers were kept at room temperature for 2 hours. The containers were then placed in a freezer at -80°C for 2 hours. Subsequently, the scaffolds were lyophilized for 2 days and removed from the containers. To remove the EDAC/NHS residue, the lyophilized silk sponge was suspended in 10 mL of quenching solution (5:1 mixture of a 0.25 M NaHSO_3 solution and 0.5 N H_2SO_4), and toroidal disk scaffolds were formed out of the porous material using a scalpel with an outer diameter of 8 mm, inner diameter of 3.5 mm and a height of 2~3 mm. The silk sponges were submerged in 70% EtOH for sterilization in preparation for cell cultivation experiments after washing with distilled water for 1 day. Before cell seeding, the scaffolds were conditioned overnight with the culture medium (Fig. 1b).

2.5. *In vitro* cultivation of silk scaffolds

To generate the AF tissues, AF cells were seeded in the toroidal disk scaffolds with lamellar and porous features and then transferred to six-well culture plates and cultured in AF cell culture media for 2 weeks. Media consisted of DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% antibiotic-antimycotic (Gibco), and 50 $\mu\text{g/mL}$ ascorbic acid (Sigma, St Louis, MO).

2.6. Scanning electron microscopy (SEM) and confocal microscopy

The cross-sections of the scaffolds prior/post cell seeding were examined by SEM (Zeiss FESEM Supra55VP, Oberkochen, Germany). The samples were fixed for 24 hours with 0.4% glutaraldehyde and then dehydrated in a series of graded ethanols prior to coating with gold/palladium for 3 minutes before SEM observation. Auto fluorescence images of hydrated silk scaffolds were observed with a Leica TCS SP2 AOBS microscopy (Leica, Mannheim, Wetzlar, Germany) with Leica confocal software. Excitation for auto fluorescence was at 488 nm and emission was collected between 500 and 535 nm.

2.7. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis was performed with a JASCO FTIR 6200 Spectrometer (JASCO, Tokyo, Japan), equipped with a deuterated triglycine sulfate detector and a multiple-reflection, horizontal MIRacle ATR attachment (using a Ge crystal). For each measurement, 32 scans were coded with resolution 4 cm^{-1} , with the wave number ranging from $400\text{--}4000\text{ cm}^{-1}$. Fourier self-deconvolution (FSD) of the infrared spectra covering the amide I region ($1595\text{--}1705\text{ cm}^{-1}$) was performed by Opus 5.0 software. Deconvolution was performed using Lorentzian line shape with a half-bandwidth of 25 cm^{-1} and a noise reduction factor of 0.3. FSD spectra were curve-fitted to measure the relative areas of the amide I region components.

2.8. Cell viability in 3D scaffolds

Cell viability in silk scaffolds was screened using a live/dead kit (Molecular Probe, Eugene, OR). Following the manufacturer's instructions, the *in vitro* sample was treated in a solution for 40 minutes. The solution is a mixture of three components: 2 mM ethidium homodimer-1, phosphate buffered saline (PBS), and 4 mM calcein AM. After washing in sterilized PBS, the sample-embedded slide was observed with a Leica TCS SP2 AOBS microscope with Leica confocal software. The region of interest was selected from z-plane

images to include either the surface or the internal pores, beginning with a bottom section at least 1 mm above the surface of the scaffolds. Depth projection micrographs were obtained from 20 horizontal sections imaged at a depth distance of 50 μm from each other. Live cells were visualized in green and dead cells in red. Viability of cells was measured by dividing the number of viable cells (green cells) with that of total cells (green cells + red cells), determined using Image J.

2.9. Histological analysis

After macroscopic observation, tissues were fixed with 4% formalin for 24 hours. These were then embedded in paraffin and sectioned in 4 μm thick slices. Serial sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry was also carried out to screen for expression of type I collagen as the major ECM protein of AF. The sections were washed sequentially in 70% ethanol and PBS and treated with 3% H_2O_2 in PBS, and 0.15% Triton X-100 was added. Once blocked with 1% bovine serum albumin (BSA) solution, they were reacted with a monoclonal antibody raised against porcine type I collagen (1:200, Chemicon, Temecula, CA) for 1 hour, followed by addition of a biotinylated secondary antibody. The protein was then detected using a horseradish peroxidase-conjugated avidin system (Vector Laboratories, Burlingame, CA). The immunostained sections were counterstained with Mayer's hematoxylin (Sigma, St. Louis MO) before microscopic examination with a Leica DMIL light microscope (Wetzlar, Germany).

2.10. Biochemical assays for DNA, GAGs and collagen content

The recovered samples ($n=4$) for DNA and GAGs were digested for 16 hours with papain solution (125 $\mu\text{g/mL}$ of papain, 5 mM L-cystein, 100 mM Na_2HPO_4 , 5 mM EDTA, pH 6.2) at 60 $^\circ\text{C}$. DNA content was measured using the PicoGreen DNA Assay according to the protocols of the manufacturer (Molecular Probes, Eugene, OR). After centrifugation, a 25 μL aliquot of supernatant was taken from each sample and placed into 96 well plates with each well containing 75 μL of 1x TE buffer. A standard curve was generated using lambda phage DNA in 0, 2.5, 5, and 10 $\mu\text{g/mL}$ concentrations. One hundred μL of a 1:200 dilution of Quant-iT PicoGreen reagent was added to each well and read using a fluorimeter with an excitation wavelength of 480 nm and an emission wavelength of 528 nm. Total GAG content was analyzed using a 1, 9-dimethylmethylene blue (DMB) assay (Whitley *et al.*, 1989). Individual samples were mixed with the DMB solution and the absorbance was measured at 525 nm. Total GAG of each sample was extrapolated using a standard plot of shark chondroitin sulfate (Sigma, St Louis, MO) in the range of 0–100 $\mu\text{g/mL}$.

The tissue engineered constructs were digested with pepsin solution (1 mg/mL of pepsin, pH 3.0) at 4 $^\circ\text{C}$ for 48 hours to determine total collagen content. Total collagen was measured as we have previously reported (Park *et al.*, 2005). A dye solution (pH 3.5) was prepared with Sirius red dissolved in picric acid saturated solution (1.3%, Sigma) to a final concentration of 1 mg/mL. The digested samples were dried at 37 $^\circ\text{C}$ in 96-well plates for 24 hours and then reacted with the dye solution for 1 hour on a shaker. The samples were then washed five times with 0.01 N HCl and the dye sample – complex in each well was resolved in 0.1 N NaOH and absorbance read at 550 nm (Versa MAX, Molecular Devices, Sunnyvale, CA). Total collagen in each sample was extrapolated using a standard plot of bovine collagen (Sigma) in the range of 0–500 $\mu\text{g/mL}$.

2.11. Real time PCR

Cultured scaffolds ($N=4$ per group) were transferred into 2 mL plastic tubes and 1.0 mL of Trizol was added. Scaffolds were chopped with micro scissors on ice. The tubes were centrifuged at 12,000 g for 10 minutes and the supernatant was transferred to a new tube. Chloroform (200 μL) was added to the solution and incubated for 5 minutes at room

temperature. Tubes were again centrifuged at 12,000 *g* for 15 minutes and the upper aqueous phase was transferred to a new tube. One volume of 70% ethanol (v/v) was added and applied to an RNeasymini spin column (Qiagen, Hilden, Germany). The RNA was washed and eluted according to the manufacturer's protocol. The RNA samples were reverse transcribed into cDNA using oligo (dT)-selection according to the manufacturer's protocol (High Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA). Collagen type I α 1 (Col I α 1), and aggrecan levels were quantified using the Mx3000 Quantitative Real Time PCR system (Stratagene, La Jolla, CA). All data analysis employed the Mx3500 software (Stratagene) based on fluorescence intensity values after normalization with an internal reference dye and baseline correction. Differences in gene expression were evaluated using the comparative Ct method (Ct [Δ][Δ] Ct comparison). Ct values for samples were normalized to an endogenous housekeeping gene. PCR reaction conditions were 2 minutes at 50 °C, 10 minutes at 95 °C, and then 50 cycles at 95 °C for 15 seconds, and 1 minute at 60 °C. The data were normalized to the expression of the housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) within the linear range of amplification and differences (Kim *et al.*, 2005). The GAPDH probe was labeled at the 5' end with fluorescent dye VIC and with the quencher dye TAMRA at the 3' end. Primer sequences for the porcine GAPDH gene were: forward primer 5'-TCG GAG TGA ACG GAT TT GG-3', reverse primer 5'-CCA GAG TTA AAA GCA GCC CT-3', probe 5'-ACG CAG TCC TCT CCA GTG GCG AA-3'. Primer sequences for the porcine collagen type I α (Col I α 1) gene were: forward primer 5'-AGA AGA AGA CAT CCC ACC AGT CA-3', reverse primer 5'-AGA TCA CGT CAT CGC ACA ACA-3', probe 5'-AAC GGC CTC AGG TAC CAT GAC CGA-3'. Primer sequences for the porcine aggrecan gene were: forward primer 5'-CCC AAC CAG CCT GAC AAC TT-3', reverse primer 5'-CCT TCT CGT GCC AGA TCA TCA-3', probe 5'-ACG CAG TCC TCT CCA GTG GCG AA-3'. Probes were purchased from Assay on Demand (Applied Biosciences, Foster City, CA).

2.12. Mechanical strength

Tensile tests were performed on an Instron 3366 testing frame (Grove City, PA) equipped with a 10N capacity load cell and BiopulsTM pneumatic clamps. The selected toroidal disk scaffolds (height, 2 mm) were hydrated in 0.1M PBS for >30 minutes, anchored to custom submersible ring-testing grips, and submerged in temperature-controlled Bioplus bath (37 $\pm$ 0.3°C) filled with PBS for at least 5 minutes prior to testing. A displacement control mode was used, with a crosshead displacement rate of 1 mm $\cdot$ min⁻¹. Taking the initial gauge length as the nondeformed average diameter ($D_0 + D_{in}/2$), the original cross sectional area (4.5 mm²) and the effective material cross sectional area (9.0 mm²) determined based on an assumed uniform and constant wall thickness (WT=2.25mm) and measured sample heights (T=2 mm), the tensile stress and strain were graphed and the initial "linear elastic modulus", elongation to failure, and ultimate tensile strength determined. The initial "linear elastic modulus" was calculated by using a least-squares' (LS) fitting between 0.1N load (porous scaffolds) or 0.01N load (lamellar scaffolds) and 10% strain past that point. Ultimate tensile strength (UTS) was determined as the highest stress value attained during the test. The elongation to failure was determined as the last data point before a >10% decrease in load (failure strain minus the strain corresponding to 0.1N load noted earlier). The toroid disk scaffolds were stretched through an elliptical shape and eventually fully stretched to a linear shape.

2.13. Statistical analysis

Statistical differences in biochemical and mechanical quantitative analysis were determined using a Mann-whitney U test (Independent t-test, SPSS) Statistical significance was assigned as **p*<0.05, ***p*<0.01 and ****p*<0.001, respectively.

3. Results

3.1. Scaffold features

The first group of scaffolds possessed lamellar features similar to the AF region of the native IVD (Fig. 2a). The other group consisted of porous spongy scaffolds that are widely used in the literature and served as a control (Fig. 2b). Observations of silk scaffolds by SEM and confocal microscopy (hydration) revealed that the inter-lamellar distance in the lamellar scaffolds was 150~250 μm and the average pore sizes of the porous scaffolds was 100~250 μm . FTIR analysis of the silk scaffolds showed characteristic peaks for silk II (the beta sheet crystalline state) at 1701 and 1623 cm^{-1} (amide I) (Kim *et al.*, 2005). Lamellar silk scaffold structures were achieved by freeze-drying and water annealing. EDAC/NHS doped silk gel was freeze-dried with water annealing to generate the porous scaffolds. Both scaffolds showed high β -sheet content after water annealing. Crystallinities of lamellar and porous scaffolds were 38% and 46%, respectively. EDAC/NHS led to a slight increase in crystallinity (~8%) of the porous scaffolds. However, EDAC/NHS did not induce β -sheet formation before water annealing (Fig. 3).

3.2. Cell morphology

Based on analysis of the confocal images, the seeded porcine AF cells were supported in the lamellar silk scaffolds over 2 weeks. The proliferating cells spread along the lamellar walls (Fig. 4a and b), while cells on the porous scaffold could not penetrate into the interior from the surface of the scaffold (Fig. 4d and e). Most of the attached and proliferated cells (>90%) survived in the both scaffolds as shown by the live/dead staining (Fig. 4c and f).

3.3. Histology and immunohistochemistry analysis

For the identification of cell distributions and AF-specific ECM molecules, thin sections of each specimen were stained with H&E and for type I collagen. Cells seeded in the lamellar silk scaffolds showed homogeneous distributions and proliferation following each wall after 2 weeks (Fig. 5a). In contrast, cells seeded in the porous silk scaffolds appeared to spread more prominently along the surfaces of the scaffolds (Fig. 5b). From type I collagen staining, cells synthesized ECM for AF tissue in both types of scaffolds. In particular, type I collagen staining was homogeneously distributed within the entire lamellar structure, while the porous scaffolds showed more staining at the scaffold surface (Fig. 5c and d).

3.4. Quantification chemical assay

DNA, GAGs, and total collagen content in the lamellar scaffolds were significantly higher than in the porous scaffolds. The DNA content in the porous silk scaffold increased slightly over time, while in the lamellar scaffolds the increase was significant at 1 and 2 weeks (Fig. 6a). In addition, the lamellar silk scaffolds showed increasing GAGs and collagen throughout the culture period. The GAG content was 5 times higher after 2 weeks than the porous silk scaffolds. The amount of GAGs ($\mu\text{g/g}$ wet weight) in the lamellar and porous silk scaffolds at 2 weeks were 771.2 ± 10.8 and 173.3 ± 74.3 , respectively ($p < 0.001$) (Fig. 6b). The total collagen content ($4.1 \pm 0.1 \mu\text{g/mg}$ wet weight) of the lamellar silk scaffolds was 2 times higher than the value in the porous scaffolds ($2.1 \pm 0.3 \mu\text{g/mg}$) (Fig. 6c).

3.5. Real time PCR analysis

To further support the results from histological observation, transcript levels related to AF differentiation markers Col Ia1 and aggrecan were analyzed. The mRNA levels of Col Ia1 and aggrecan were significantly increased in the lamellar silk scaffold with the time in culture. In contrast, mRNA levels of these genes did not significantly increase in the porous

silk scaffolds, while they were around 2 times higher in the lamellar silk scaffolds after 2 weeks (Fig. 7).

3.6. Mechanical strength

Scaffolds from cell culture were evaluated for their mechanical properties. The porous scaffolds had a higher linear elastic modulus and ultimate tensile strength at day 1, while both the lamellar and porous scaffolds showed similar values after 2 weeks in culture (Fig. 8a and b). There were no significant differences in elongation to failure between the lamellar and porous scaffolds (Fig. 8c).

4. Discussion

Although degenerative IVD disease constitutes a large healthcare problem, in surgical treatments for IVD diseases, degenerated AF tissues showed little regeneration due to its avascular structure (Goins *et al.*, 2005). Thus, tissue engineering of the AF is a potential option for IVD repair. The AF consists of a series of loosely connected concentric layers (lamellae) of highly oriented type I collagen tissue that encloses the NP (O'Halloran and Pandit 2007). In tissue engineering aimed at functional tissue restoration, the scaffold plays an important role as a functional template that guides the cellular remodeling process and can potentially provide the cells with temporary protection from unfavorable local implantation environments (Gruber *et al.*, 2004). Various biomaterials have been explored for AF tissue replacement (Gruber *et al.*, 2006, Sato *et al.*, 2003, Shao and Hunter 2007). However, these systems have not met the requirements for AF structure and mechanical properties.

Silk scaffolds utilized in the present study have shown promise in bone and cartilage tissue engineering *in vitro* and *in vivo* due to their impressive mechanical properties, biocompatibility and biodegradability (Hofmann *et al.*, 2006, Kim *et al.*, 2007). Two types of scaffold morphologies formed from silk were studied in the present work. The first mimics the lamellar features of the native IVD associated with the AF region. The second was a porous spongy scaffold that is widely cited in the literature for these types of tissue systems, and served as a control in the present study. Toroidal scaffolds were formed out of the lamellar and porous materials. Thus, morphological features were addressed with scaffold designs, to emulate the AF.

To generate the lamellar structure of AF tissue, in prior studies alginate fiber was used in combination with a rotating spinner flask (Shao and Hunter 2007). In the current paper, the fabrication of a lamellar structure for AF tissue was accomplished using a bottle freeze-drying technique. In initial trials, lamellar shapes from pure silk solution were generated by a simple freeze drying technique. However, inter-lamellar distance (10~20 μm) was too narrow for adequate cell seeding and function. To generate larger inter-lamellar distances, 0.2% sodium alginate was mixed into silk solution which led to 150~250 μm inter-lamellar distances, due to different ice crystal sizes during freezing (Fig. 2). Based on our scaffold preparation method, the lamellar structures in this study were not circumferentially oriented. Circumferentially oriented structures are assumed to be important for the functional restoration of AF tissue. Therefore, further methods development will be needed to fully recapitulate this complex architecture..

In order to fabricate porous silk scaffolds, we have previously used a salt leaching approach. However, small porous (<250 μm) shapes were difficult to generate by salt leaching. As an alternative technique, EDAC/NHS was added into the silk solution in the present study. As a result, EDAC/NHS mixed silk solution formed a gel after 2 hours, due to the ability of EDAC/NHS to induce crosslinking between amine functional groups on the amino acids in

the silk protein chains. The added EDAC/NHS resulted in only a small increase in crystallinity after water annealing and produced uniform 100~250 μm pore (Fig. 3).

The lamellar shaped scaffolds supported cell seeding and proliferation to form an AF-like tissue. SEM images showed even distribution of cells in the lamellar scaffolds. After 2 weeks, entire areas of the walls were covered by proliferating cells. Thus the cells could penetrate into the inside of the scaffold. In contrast, porous silk scaffolds showed proliferating cells mainly on the surface of the scaffolds (Fig. 4). Low cell penetration on the porous silk scaffold was already previously reported; 150~250 μm pores in silk scaffolds showed non-uniform distribution of tissue growth throughout the structure (Chang *et al.*, 2007). In follow up studies large pore scaffolds (600 μm) and spinner flask dynamic culture were used to successfully overcome this shortcoming (Chang *et al.*, 2010). In a different study, a collagen honeycomb-shaped scaffold was used to retain cells for better tissue formation (Sato *et al.*, 2003). However, these porous scaffold based studies did not mimic the AF structure. Our study to mimic the AF lamellar structure served two functions: (i) simulation of the lamellar structure of the native tissue and (ii) support of cell penetration into the scaffold interior. Seeded AF cells were able to proliferate between the lamella walls in the scaffold (Fig. 5).

A successful scaffold provides physical support for cell attachment and promotes cell proliferation and desired ECM deposition (Shao and Hunter 2007), as the physiological properties of the disc are linked to the composition of its ECM (O'Halloran and Pandit 2007). The major components of the AF ECM are fibrillar collagens and proteoglycans (PG) (Kluba *et al.*, 2005). The largest and most important PG in the disc matrix is aggrecan which consists of a protein core with attached glycosaminoglycans (negatively charged chondroitin sulfate and keratan sulfate) (Ghosh *et al.*, 1980). The chondroitin sulfate molecule plays a crucial role in retaining water, which in turn gives the disc its resilient compressive strength (Meisel *et al.*, 2007). When AF cells were seeded onto the lamellar or porous silk scaffolds, AF cells adhered to both scaffolds and synthesized collagen and proteoglycans. The lamellar scaffolds supported more AF tissue specific features, based on histological, biochemical and gene expression data for collagen and aggrecan (Fig. 6 and 7).

AF tissue should be mechanically stable as IVDs are particularly vulnerable to fatigue failure, especially when the bending moment is high (Aubin *et al.*, 2004). Failure of the AF or damage to the collagenous network is a potential cause of disc herniation. With disc degeneration, failure stresses were significantly reduced (Acaroglu *et al.*, 1995). This mechanical property of human AF tissue is reported at 0.9~3.8 Mpa to failure strength, 20~60 % strain and 0.4~0.5 Mpa tensile modulus. (Fujita *et al.*, 1997, Green. *et al.*, 1993, Iatridis *et al.*, 2005). Silk scaffolds were tested in the elongation to failure mode in this study and the lamellar and porous scaffolds showed similar properties to that of the native tissue. In measuring linear elastic modulus and ultimate tensile strength (UTS), lamellar scaffolds showed weaker properties than the porous scaffolds at initial time frames, while the values did not show statistical difference after 2 weeks (Fig. 8a and b). Although the testing method used in the present study measured the mechanical properties while maintaining the original structure, this can be a highly complex non-linear test of the structural stiffness if we consider the deformation of the toroid scaffolds itself. However, the force used for toroid scaffold deformation was negligible (less than 0.1 N for porous and 0.01 N lamellar scaffolds) under PBS hydrated measuring conditions compared to the force needed for stretching the scaffolds. Therefore, linear modulus and strength are reflected in the data shown, while additional more complex mechanical tests could be considered in future studies.

5. Conclusions

Even though the lamellar silk scaffolds were not circumferentially oriented, the lamellar shape architecturally resembled native AF. This study suggested a method to fabricate lamellar structure using silk material and to provide large dimensions for cell proliferation and production of ECM. In addition, histological and biochemical analyses, immunohistochemistry, and gene expression profiling revealed time-dependent development of AF phenotype from the seeded cells. The cells within the lamellar scaffolds maintained the architecture of a native AF over two weeks of culture. Ultimately this tissue mimetic using lamellar silk scaffolds for the AF provide structure and function, with the current results providing a foundation for further study toward biphasic tissue engineered IVD tissue.

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Fig. 1. Methods for scaffolds fabrication: (a) lamellar shaped scaffold, (b) porous shaped scaffold.

Fig. 2. Structure of scaffolds: (a) lamellar silk scaffold, (b) porous silk scaffold. Scale= 200 μm

Freeze-dried silk scaffolds	β -sheet / %
Freeze-dried	17 %
Freeze-dried w/ EDAC/NHS	17 %
Freeze-dried - water annealed	38 %
Freeze-dried w/ EDAC/NHS - water annealed	46 %

Fig. 3. FTIR spectra of silk scaffolds: (a) Freeze dried scaffold (b) Freeze dried scaffold with EDAC/NHS (c) Freeze dried scaffold – water annealed (lamellar structure) (d) Freeze dried scaffold with EDAC/NHS–water annealed (porous structure)

Fig. 4.

SEM images and live cell staining of porcine AF cells on lamellar and porous scaffolds. Lamellar scaffolds at (a) 1 week, (b) 2 weeks. Porous scaffolds at (d) 1 week, (e) 2 weeks. Scale bars = 100 μm . Live/dead staining of seeded AF cells on: (c) lamellar and (f) porous scaffolds. Arrow indicates proliferating cells. Scale bars = 300 μm .

Fig 5.
H&E staining for cells after 2 weeks: (a) lamellar scaffolds, (b) porous scaffolds.
Immunohistochemistry for type I collagen, (c) lamellar scaffolds, (d) porous scaffolds.
Dashed arrows indicate cells. Extracellular matrix stained positive for type I collagen with brown color (solid arrow). Scale bars = 100 μm

Fig 6. Chemical analysis: (a) DNA content, (b) GAGs, (c) total collagen content per scaffold. Data shown as mean $\pm$ standard deviation from 4 samples (*p<0.05, **p<0.01 and ***p<0.001).

Fig 7. Transcript levels related to AF tissue differentiation markers: (a) *Colla1* and (b) *aggrecan*. Data quantified by real-time PCR and normalized to GAPDH within the linear range of amplification. Data shown as mean $\pm$ standard deviation from N=4, *p<0.05, **p<0.01 and ***p<0.001.

Fig. 8. Analysis of mechanical strength at 1 day and 2 weeks: (a) Linear elastic modulus (b) Ultimate tensile strength, (c) Elongation to failure between lamellar and porous silk scaffolds.