Oriented lamellar silk fibrous scaffolds to drive cartilage matrix orientation: Towards annulus fibrosus tissue engineering

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A B S T R A C T
A novel design of silk-based scaffold is developed using a custom-made winding machine, with fiber alignment resembling the anatomical criss-cross lamellar fibroin fibre orientation features of the annulus fibrosus of the intervertebral disc. Crosslinking of silk fibroin fibers with chondroitin sulphate (CS) was introduced to impart superior biological functionality. The scaffolds, with or without CS, instructed alignment of expanded human chondrocytes and of the deposited extracellular matrix while supporting their chondrogenic redifferentiation. The presence of CS crosslinking could not induce statistically significant changes in the measured collagen or glycosaminoglycan content, but resulted in an increased construct stiffness. By offering the combined effect of cell/matrix alignment and chondrogenic support, the silk fibroin scaffolds developed with precise fiber orientation in lamellar form represent a suitable substrate for tissue engineering of the annulus fibrosus part of the intervertebral disc.

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1. Introduction

The cellular alignment and architectural organization of the fibrous extracellular matrix (ECM) of a tissue play fundamental roles in its biomechanical properties. The multilamellar, fiber-reinforced composite structure of the intervertebral disc (IVD) provides shock-absorbing capabilities to the spinal column and imparts flexibility between adjacent vertebrae. The annulus fibrosus (AF) part of the IVD consists of 15–25 concentric layers; each layer is reinforced by collagen fibers aligned at an approximately 30° angle with respect to the transverse plane of the disc, but in alternate directions in successive layers [1]. This cross-aligned, fiber-reinforced organization is critical for proper biomechanical functioning of the AF. It allows the conversion of compressive force to lateral force to withstand extrinsic tensile stresses (circumferential, longitudinal and torsion), and ultimately makes the spine flexible enough to bend and twist in all directions.

Orientation is evident even from the early stages of disc development. In the embryonic stage, longitudinally aligned collagen fibers radiate into the cartilaginous layer of the primordial vertebral body (the predecessor of Sharpey’s fibers), along which AF precursor cells and the other deposited ECM components become precisely oriented [2,3]. Age-dependent dehydration of the nucleus pulposus (NP) and crack development in the AF layers [4], probably caused by a number of factors such as metabolic changes, genetic predisposition and biomechanical issues [5], lead to degeneration of the disc. A reduction in the diameter and increased fibrillation of collagen fibers [6], the disorientation of fiber alignment and a decline in ECM turnover are hallmarks of IVD degeneration.

Replacement of degenerated disc by a tissue engineered substitute could offer major advantages over arthroplasty or the implantation of a prosthetic disc, in terms of the initial matching of biomechanical properties and adaptive remodeling over the long term. Recently, several attempts have been made to engineer AF tissues using scaffolds with a variety of chemical compositions and architectures [5,7–12]. However, none of these studies could successfully simulate the precise anatomical orientation of collagen fibers in lamellar multilayered AF tissue. As a result, the mechanical properties of most of these engineered tissues were several orders of magnitude below the stiffness of IVD, especially under tension and compression, and would therefore be expected to provide insufficient mechanical support after implantation at the intervertebral joint site.
Simulation of collagen fibers orientation could be the key feature of reconstructing AF tissue layers and governing their mechanical anisotropic behavior. The development of a scaffold having custom-made fiber alignment might allow cells to deposit fibrous ECM proteins at a desired orientation and ultimately ensure the overall biomechanical functions of the tissue. Contact guidance for cell orientation and alignment has previously been demonstrated in two-dimensional (2-D) electrospun matrices for engineering of myotubes based on myoblasts [13,14] and for AF engineering based on mesenchymal stem cells [15–17]. However, the electrosprinning approach is often associated with a number of limitations, such as low porosity that does not allow uniform cell infiltration, a planar 2-D culture system and a mismatch of mechanical strength of nanofibers compared to discrete, robust collagen fibers [15]. To achieve a closer approximation of the structurally oriented AF tissue architecture, 3-D scaffolds were produced using either oriented fibers alone [18] or in combination with hydrogels [19]. However, the latter approach of using fiber-hydrogel composite [19] failed to create alternating angled fibrous orientation in successive layers, and as a result could not simulate the complexity of the AF tissue’s specific microenvironment.

In an attempt to generate a full disc substitute, Park et al. [20] reported a biphasic porous lamellar biomaterial using silk scaffolds for AF engineering, in combination with fibrin/hyaluronic acid gels for the NP. These 3-D scaffolds could support marginal cartilaginous matrix formation, but still cold not achieve the particular anatomical cellular orientation required to fulfill the mechanical requirements.

Chondroitin sulfate (CS) is known to play a critical role in disc morphogenesis, as evidenced by the highly specific distributions throughout the embryonic development, postnatal growth and aging stages [21]. It is associated with the glycosaminoglycan (GAG) chains of the matrix proteoglycan of the AF tissue matrix [22], and can retain a number of molecules, such as growth and differentiation factors, enzymes and chemokines, owing to sulfation motifs. Varghese et al. [23] previously showed that CS attached to poly(ethylene glycol) hydrogels increased the chondrogenic differentiation of bone marrow stromal cells by enhancing pre-chondrogenic clustering of mesenchymal cells and up-regulating the expression of cartilage-specific genes. In addition, CS exhibits anti-inflammatory activity [24], chondroprotective properties [25] and therapeutic effects on osteoarthritis [26], which may help to restore degenerated disc functionality.

The purpose of the present study was to investigate the effects of fiber alignment and presentation of CS on the in vitro deposition and orientation of a cartilaginous matrix. Thus, we first developed a process to manufacture a unique custom-made silk fibroin fibrous scaffold having precisely oriented macrodiameter silk fibers, simulating the structural hierarchy and lamellar architecture of AF tissue. Some of the silk fibrous scaffolds were then modified by crosslinking with CS. Human nasal chondrocytes were cultured onto these 3-D scaffolds and their chondrogenic redifferentiation and matrix deposition were investigated. Nasal chondrocytes were chosen as the cell source as they have previously been reported to exhibit some reproducible capacity to generate cartilaginous tissues after expansion, unlike other chondrogenic cells [27]. Moreover, they share the same neuro-ectodermal origin of intervertebral disc cells [5] and are responsive to physical forces resembling joint loading [28].

2. Experimental

2.1. Design of a custom-made winding machine

Movements of the custom-made silk winding machine were controlled by a rotating assembly and a sliding assembly. The two assemblies were placed diagonally to each other and contained a DC motor having a variable speed of 0–40 rpm. The rotating assembly consisted of a stainless steel rod that maintained the angle of silk fiber winding, which could be adjusted according to the scale attached to it. The silk hydrogel was mounted on this rotating mandrel. The sliding assembly was used to manipulate the direction of winding. It consisted of a sliding block linked to a pair of microswitches to reverse the direction of rotation of the motor, which in turn controlled the direction of winding in the forward and reverse directions, resulting in a criss-cross pattern of fiber winding in successive layers. The entire unit was controlled by a control panel, which ensured the smooth winding sequence and the safety of the motor during operation.

2.2. Preparation of silk fibroin scaffolds

Anthera mylitta silk fibers were obtained from local textile industry and degummed by boiling in 0.02 M Na2CO3 for 30 min to remove the outer sericin layer of silk. The remaining silk fibroin fibers were extensively rinsed in milli-Q water. The fibers were then dried and scaffolds were prepared using the custom-made winding machine.

For the preparation of fibroin hydrogel, Anthera mylitta coconos were boiled for 30 min in 0.02 M Na2CO3 and then rinsed with water to extract sericin. The silk was dissolved in 9.3 M LiBr solution, yielding a 7–8% (w/v) solution, which was dialyzed in water using Slide-a-Lyzer dialysis cassettes (Pierce, MWCO 3500). After obtaining the pure silk fibroin dialyzed solution, methanol was added and it was allowed to stand. The methanol treatment induces beta-sheet formation within the silk fibroin and resulted in gelation. With the help of a biopsy punch, scaffolds were cut into the required dimensions. Silk hydrogel was placed within the central mandrel of the winding machine, and the silk fiber bobbin was mounted on a spindle placed on one end of the machine. The silk fiber from this bobbin passed through the keyhole in the sliding block and was wrapped around the hydrogel mounted on the rotating mandrel in such a way that the silk fibers lay parallel to each other at an angle of approximately 30° to the vertical axis in one layer and oriented in the opposite direction in successive layers. Cylindrical scaffolds (5–6 mm thick) were excised from the main cylindrical structure using a surgical knife. The silk scaffolds were then sterilized by autoclaving.

2.3. Crosslinking with CS

CS (from bovine trachea, MW 105 Da, Sigma) was first oxidized using periodate salt (Fig. 1A and B). Briefly, 600 mg of CS and 616 mg of NaIO4 were dissolved in 10 ml of deionized water whilst being protected from light. The reaction was allowed to continue for 1 h in the dark, with vigorous stirring at 40 °C. The reaction product was purified by filtration with Sephadex G-25 (Sigma) size-exclusion chromatography. Prior to the second step of the reaction, degummed silk fibers were rinsed for 1 h with sodium acetate solution (10% w/v). The fibers were then thoroughly washed with deionized water, dried and subjected to crosslinking with oxidized CS. In a typical reaction, 1 g of the silk fibers was immersed in 25 ml of 6% (w/v in distilled water) oxidized CS solution. Crosslinked fibers were thoroughly washed in water to ensure that there was no unbound CS in the final product.

2.4. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

Infrared spectra for CS, oxidized CS, silk fibroin and silk-CS were measured by ATR-FTIR on an Alpha-P spectroscope (Bruker). All
spectra were taken in the spectral range of 4000–500 cm\(^{-1}\) using an accumulation of 264 scans with a resolution of 4 cm\(^{-1}\).

2.5. Nuclear magnetic resonance (NMR)

All NMR experiments were carried out at 25 °C on a Bruker Avance III spectrometer equipped with a cryogenic triple-resonance probe, operating at a field strength of 500 MHz. Temperature calibration was performed using a 100% d4-methanol sample [29]. One-dimensional \(^1\)H-NMR and 2-D \([^{15}\text{N,}^1\text{H}]\) heteronuclear single quantum coherence (HSQC) spectra in a natural abundance of \(^{15}\text{N}\) were measured for oxidized CS, silk fibroin and silk-CS. All measurements, processing and analysis of NMR spectra were performed using TOPSPIN 2.1 (Bruker AG).

2.6. Cell culture

Human nasal cartilage was harvested from three human donors (A, B, C) aged between 25 and 54 years old (one male and two females), following approval by the local ethical committee and with informed consent at University Hospital, Basel. Chondrocytes were isolated after 22 h of incubation at 37 °C in 0.15% type II collagenase and resuspended in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U ml\(^{-1}\) penicillin and 100 μg ml\(^{-1}\) streptomycin (complete medium). Chondrocytes were plated in tissue culture dishes at a density of 10^6 cells cm\(^{-2}\) and expanded in complete medium supplemented with 5 ng ml\(^{-1}\) fibroblast growth factor-2 and 1 ng ml\(^{-1}\) transforming growth factor β1, as previously described [30]. When cells were subconfluent, they were detached by sequential treatment with 0.3% type II collagenase and 0.05% trypsin/0.53 mM EDTA, and replated at 5 × 10^3 cells cm\(^{-2}\).

Prior to cell culture, 3-D fibroin fibrous scaffolds without hydrogel were sterilized by overnight incubation at 70% ethanol and pre-wetted in complete medium. Chondrocytes were seeded statically at a density of 7.0 × 10^7 cells cm\(^{-3}\). Constructs were statically cultured in complete medium supplemented with 10 μg ml\(^{-1}\) insulin, 0.1 mM ascorbic acid 2-phosphate and 10 ng ml\(^{-1}\) transforming growth factor β3. The medium was changed twice a week. Constructs were harvested for analysis after 1 day, 2 weeks and 4 weeks.

2.7. Cell seeding efficiency

The efficiency of cell seeding was calculated by measuring the DNA content in constructs 1 day after seeding and comparing it to that of the initial number of cells seeded.

2.8. MTT staining

Cell viability was determined using an MTT assay. Constructs harvested after 1 day were rinsed in phosphate-buffered saline (PBS) and stained with the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for 3 h at 37 °C. Photographs were taken to record the intensity of violet staining indicative of the presence of metabolically active cells.
2.9. Biochemical analysis

Constructs were digested with proteinase K solution. The GAG content was measured spectrophotometrically after reaction with dimethylthelylene blue, with chondroitin sulfate as a standard. The GAG content was normalized to the DNA amount, which was measured using a CyQUANT cell proliferation assay kit (Molecular Probes, Eugene, OR, USA), with calf thymus DNA as a standard.

The amounts of type II collagen were assayed by inhibition ELISA using a mouse IgG monoclonal antibody to denatured collagen II [31]. Total collagen was measured as hydroxyproline using a Biochrom20 Plus amino acid analyzer [32].

2.10. Histological and immunohistochemical analysis

Human AF tissue was obtained after dissection of a human degenerated disc obtained from a 56-year-old female patient with informed consent at University Hospital, Basel. The tissue was fixed in 10% formalin and stained with hematoxylin and eosin (H&E).

Constructs were rinsed with PBS, fixed in 4% formalin, embedded in paraffin, cross-sectioned (7 μm) and stained either with H&E for studying cell morphology or with Safranin-0 for sulfated glycosaminoglycans GAG. Sections of constructs were processed for immunohistochemistry using antibodies against type I collagen (Quartett, Berlin, Germany) and type II collagen (II-16B3, Hybridoma Bank, University of Iowa, USA) [33].

2.11. Scanning electron microscopy

Constructs were harvested after 1 day, 2 weeks and 4 weeks, and fixed in 2% glutaraldehyde. The samples were dehydrated using gradients of ethanol and sputter-coated with gold. All samples were evaluated using a scanning electron microscope (SEM EVO).

2.12. Transmission electron microscopy

Constructs were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, at 37 °C, then stored at 4 °C. They were rinsed in 0.1 M phosphate buffer twice for 15 min at 4 °C, and post-fixed in 1% osmium tetroxide for 2 h at 4 °C. Samples were then washed in 0.1 M phosphate at 4 °C, dehydrated in graded acetone and finally embedded in Epon 812 resin. Sections were cut to 30–90 nm using a diamond knife on a Reichert-Jung Ultracut E microtome, stained with 2% uranyl acetate and viewed with a Morgagni 268D electron microscope (Fei Electron Optics, Holland).

2.13. Biomechanical characterization of constructs

The biomechanical properties of the engineered constructs derived from cells of donor C were characterized by uniaxial, radi-cally unconstrained compression mode at 1 mm min⁻¹ using specimens of constructs based on silk and silk-CS scaffolds, and of samples of the outer annulus of mature goat caudal discs (as control) of similar dimensions. Goat discs were collected from a nearby slaughter house and the outer AF layer was separated out from the rest of each disc with a surgical knife to obtain sections having a thickness of 2 mm and a width of 5 mm. Compression tests were carried on a computer-controlled Universal Testing Machine (UTM), model H5KS (Tinus Olsen, England) with QMAT 5.37 professional software. The cross-sectional area was measured (randomly chosen) by taking three measurements of thickness using a vernier caliper. The samples (n = 10) were clamped onto the jaws of the UTM. A compressive load range of up to 900 N and a test speed of 1 mm min⁻¹ were applied in perpendicular direction to the lamellae of the constructs and AF tissues.

The Poisson’s ratio for an isotropic linearly elastic material through unconstrained compression was estimated using the following formula:

\[ (E/\rho_a) = (1 - v)/(1 + v)(1 - 2v) \]

By simplifying the above formula,

\[ \geq 2v^2 + va - a = 0 \]

where \( a = 1 - (E/\rho_a) \).

The Poisson’s ratio of the material was calculated through the positive root of this quadratic equation, where \( Ha \) is the aggregate modulus, i.e. the compressive modulus, \( F \) is Young’s modulus, i.e. the force at the point of breaking of the material in the compressive test/1000 x % of elongation (MPa), and \( v \) is Poisson’s ratio of the material.


The mechanical properties at the nanometer scale were assessed with a Nanowizard I atomic force microscope (JPK Instruments, Berlin, Germany) on engineered constructs derived from cells from donors B and C, as described earlier [34]. Briefly, standard rectangular cantilevers with sharp silicon nitride pyramidal tips (nominal tip radius ~20 nm, nominal k = 0.2 ± 0.04 N m⁻¹; CSC38/S3N4/AIBS, MikroMasch, Tallinn, Estonia) were used for stiffness measurements. Prior to the indentation testing, the spring constant of each cantilever was determined using the thermal noise method [35]. Each sample was measured at nine or more random spots comprising a scan area of 3600 μm², with an indentation force of 20 nN at a frequency of 3 Hz. Generated maps with force-distance curves of at least 16 × 16 points were analyzed using the Oliver & Pharr method for calculating the dynamic elastic modulus (stiffness) in a custom-developed Labview software [36].

2.15. Statistical analysis

Unless otherwise mentioned, data are presented as mean ± standard deviation. Means were compared using the Mann–Whitney U-test. Statistical analyses were performed using the Sigma Stat Software (SPSS Statistics 19), with p < 0.051 as the criterion for statistical significance.

3. Result

3.1. Crosslinking of CS with silk fibrin fibers

FTIR analysis was done to assess the efficacy of the crosslinking reaction. ATR-FTIR spectra of CS, oxidized CS, degummed silk fibrin and silk-CS are shown respectively in Fig. 2A–D. The broad absorption band at 3316 cm⁻¹ was found to be due to stretching vibration of hydroxyl groups ([(O–H)] and [(O=C–O)], respectively), confirming the presence of saccharide hydroxyl groups in CS. The carbonyl stretching vibration ([C=O]) of aldehyde groups (CHO) was observed at an absorption peak of 1733 cm⁻¹ (spectra B). The appearance of a band at 1733 cm⁻¹ and the decrease in intensity of the band at 1035 cm⁻¹ due to the fact that the adjacent hydroxyl groups in the CS backbone were converted to aldehyde groups in oxidized CS. The CS oxidation reaction was carried out for different time periods, ranging from 1 to 16 h. However, the intensity of the bands at 1733 and 1035 cm⁻¹ was found to be identical in all the products. Therefore, in our modified reaction condition, a period of 1 h was sufficient for CS oxidation, rather than the 16 h reported earlier [37]. Spectrum C shows the characteristic amide I ([ν(C=O)], amide II ([ν(N–H)]) and amide III ([ν(C–N)]) absorption bands, at 1636, 1511 and 1229 cm⁻¹ respectively, due to the amide groups present in silk fibrin. The broad absorption band at 1652 cm⁻¹ was found to be due to amide III ([ν(C–N)]) of oxidized CS.
might also be involved in the complex formation. We could clearly observe resonances of aromatic protons when spectra of silk fibroin were measured in 100% D2O. These aromatic protons were buried beneath the strong peaks of exchangeable –NH2 protons, which became observable when –NH2 protons were exchanged with 2H. Further, –NH2 protons resonating at 6.65 and 6.92 ppm in the 1-D 1H-NMR spectrum of oxidized CS disappeared upon complex formation with CS. Hence, the spectra were dominated by silk fibroin in the crosslinked product.

Crosslinking of silk fibroin with CS was validated using solution-state NMR spectroscopy. In the 1-D 1H-NMR spectrum of oxidized CS a characteristic very sharp peak of aldehydic proton (–CHO) was observed at 8.10 ppm [39] (Fig. 3A). This peak shifts 15 Hz upfield when CS forms a complex with silk fibroin. Further, –NH2 protons resonating at 6.65 and 6.92 ppm in the 1-D 1H-NMR spectra of silk fibroin disappear upon complex formation with CS. This clearly indicates that NH2 groups are directly involved in the complex formation. We could clearly observe resonances of aromatic protons when spectra of silk fibroin were measured in 100% D2O. These aromatic protons were buried beneath the strong peaks of exchangeable –NH2 protons, which became observable when –NH2 protons were exchanged with 2H. There were no changes in the chemical shifts of the aromatic protons upon complex formation, indicating that they were not involved in the reaction (Fig. 3A). This observation confirmed the formation of Schiff’s base between free amines of silk fibroin and oxidized CS. Hb protons of tyrosine residues of silk fibroin resonating at 2.76 and 2.89 ppm also disappeared upon complex formation (Fig. 3A). The broadening of these resonances can be attributed to a possible chemical exchange occurring at the micro- to millisecond timescale. The changes in dynamics of tyrosine rings indicate that some of its positively charged neighboring residues might also be involved in the complex formation.

We observed about eight backbone –NH peaks in the 2-D [15N,1H] HSQC spectra of silk fibroin measured with a natural abundance of 15N [Fig. 3B], which were probably from the residues located at flexible loops having short local correlation times. In the overlay of the 2-D [15N,1H] HSQC spectra [Fig. 3B] of silk fibroin (yellow), oxidized CS (red) and silk-CS (blue), one could observe the disappearance of three backbone –NH peaks (marked in circles) of silk-CS. Disappearance of these backbone –NH resonances of silk fibroin [Fig. 3B] clearly indicated their direct involvement in complex formation with CS (Fig. 1C). All other –NH peaks of CS were observed in complex with an identical chemical shift as in free oxidized CS. Hence, CS was indeed tethered to silk fibers mainly at the arginine residues or at free amine groups present in the amorphous regions of silk. No other –NH peaks were observed, due to the large size and the non-enriched 15N sample. The missing –NH resonances can be observed only if a transverse relaxation optimized spectroscopy spectrum is measured for uniformly 2H,15N-labeled silk fibroin [40], which is almost utopian at this juncture. Further arguments in favor of a effective crosslinking reaction between CS and silk fibers are that the crosslinked silk fibers became insoluble upon treatment with lithium bromide, and their color changed from colorless to deep brown.

3.2. Development of lamellar fiber orientation

In order to simulate the collagen fiber alignment and related multi-lamellar structural hierarchy of the annulus fibrosus, a rapid silk winding machine was designed and prototyped (Fig. 4A). Silk fibroin hydrogel was placed within the central mandrel (Fig. 4B) of the machine and the degummed Antheraea mylitta silk fibers (diameters in the range of 20–30 μm) were wrapped around the hydrogel in such a way that they lay parallel to each other at an angle of approximately 30° to the vertical axis in one layer and were oriented in the opposite direction in successive layers. Controlled orientation of silk fiber over a core hydrogel at alternative angles in successive layers, as visualized by SEM imaging (Fig. 4C), resulted in the precise simulation of the anatomical criss-cross lamellar features of human AF tissue. Large inter-fiber pores would facilitate infiltration of cells after seeding. Fiber tension was optimized in order to achieve the minimum amount of delamination upon distortion, bending or axial rotation.

3.3. Cell seeding efficiency and viability

Nasal chondrocytes were expanded and seeded statically onto control silk scaffolds or silk-CS scaffolds. The cell seeding efficiency
was calculated to be 66.3 ± 8.9% on the control silk scaffolds (n = 4 constructs) and 55.6 ± 11.1% on the silk-CS scaffolds (n = 3 constructs).

MTT staining of the constructs showed the presence of viable and metabolically active cells, visualized through violet staining (Fig. 5C and D). Constructs based on both types of scaffolds, silk and silk-CS, were positively stained, but direct comparison of intensity of staining was not possible due to the initial darker color of the scaffolds crosslinked with CS (Fig. 5A and B). In both groups, staining was not homogeneously distributed but, rather, was detected in the cell seeding side of the scaffold, due to the static seeding method combined with the large thickness of the scaffolds.

3.4. Effect of fiber orientation as morphological guidance

SEM analysis was performed on constructs based on control silk or silk-CS scaffolds cultured for 1 day, 2 weeks or 4 weeks (Fig. 6). After 1 day (Fig. 6A), the cells were firmly attached in both the control silk scaffolds and silk-CS fibers, and were spread out. After 2 weeks, in the presence of chondrogenic medium, cells had started the process of condensation, leading to the onset of aggregated clusters (Fig. 6B) and the production of fibrous ECM proteins. After 4 weeks, silk fibroin fibers were homogeneously covered by ECM in both the control silk scaffolds (Fig. 6C) and the silk-CS scaffolds (Fig. 6D and E). ECM proteins produced by the chondrocytes were morphologically guided by the alignment of the silk fibers (Fig. 6C and F), in both control the silk scaffolds and the silk-CS scaffolds (Fig. 6G), closely resembling the orientation of cells and ECM of human AF tissue (Fig. 6H). TEM studies were conducted in order to further validate this observation and to elucidate the correlation between the silk fiber/cell orientation and the deposition of an oriented fibrous ECM. The oval-shaped cell morphology present in constructs cultured for 4 weeks indicated the presence of redifferentiated chondrocytes (Fig. 7A and B). Cells were surrounded by extensive amounts of small scattered fibrous ECM proteins, and proteoglycan granules were present in the matrix (Fig. 7C, indicated by the arrow). The collagen fibers produced by the cells also followed the orientation of the cell alignment. The cell cytoplasm was well populated with rough endoplasmic reticulum, indicative of the synthesis of ECM proteins (Fig. 7D, indicated by the arrow).
3.5. Chondrogenic redifferentiation capacity in silk fibroin scaffolds

To assess whether expanded nasal chondrocytes could redifferentiate on silk and silk-CS scaffolds, constructs were cultured for 2 or 4 weeks in chondrogenic medium and analyzed both histologically and biochemically. When harvested after 4 weeks in culture, constructs exhibited a white, glossy appearance characteristic of cartilaginous tissues (Fig. 8A). GAG/DNA ratios were low after only 1 day of culture but had increased by up to 5.1-fold after 4 weeks (Fig. 8B). At both times of culture, these ratios were similar in constructs based on control and silk-CS scaffolds. Quantification of collagen type II and total collagen showed that, after 4 weeks in culture, the amounts of both types of proteins (expressed per DNA) were not statistically significantly different in constructs based on control or silk-CS scaffolds. Quantification of GAG was confirmed by Safranin-O staining, which showed that the intensity of staining for GAG was similar in constructs based on silk and silk-CS scaffolds (Fig. 9A and B).

Immunohistochemical analysis showed that a positive staining for collagen type I (CI) and collagen type II (CII) could be detected in constructs based on both silk and silk-CS scaffolds (Fig. 9C–F). Where cells look more fibroblastic, with an elongated shape, tissues were positively stained for CI, whereas in areas where the cells appeared more round, the matrix was stained for CII. High-magnification image of the constructs based on the silk scaffolds and stained for CI (Fig. 9G and H) showed that collagen fibers followed the orientation of the silk fibers, as can be seen close to the longitudinal sections of the fibroin fibers indicated by arrows. This result confirms the electron microscopic observations (Figs. 6 and 7), where ECM produced by the chondrocytes was shown to follow the alignment of the silk fibers.

3.6. Mechanical properties of silk fibroin-based constructs

We further investigated whether replication of the anatomic orientation of cells and ECM and the biofunctionalization of silk fibrous scaffolds with CS could result in engineered constructs with appropriate mechanical properties. The mechanical properties of the constructs generated from the cells of two donors were assessed in compression mode in the UTM and further validated by AFM tests (Fig. 10).

The compression tests of constructs generated with cells from donor C showed a 9-fold increase in compressive modulus of the constructs based on the silk or silk-CS scaffolds with time in culture from 1 day to 4 weeks (Fig. 10A). The compressive modulus after 4 weeks of culture was slightly (1.4-fold) higher in the constructs based on the silk-CS scaffolds than in those on the silk scaffolds, and was similar to that previously reported for human IVD [16,41–43]. Poisson’s ratios of constructs based on nasal chondrocytes cultured for 4 weeks on silk and silk-CS scaffolds were respectively 0.50 and 0.43, which are within the range of values measured for AF tissues in native goat IVD (0.49) (Fig. 10B).

AFM analysis of constructs using cells from two different donors also showed that the constructs based on the silk-CS scaffolds exhibited a slightly greater, though statistically significant ($p = 0.051$), stiffness than those based on silk scaffolds after 4 weeks in culture (Fig. 10C). To assess whether this greater stiffness was due to the initially greater stiffness of the scaffold itself, we examined the mechanical properties of the silk and silk-CS scaffolds without cells by AFM, and showed that, in fact, the silk fibroin scaffold was initially slightly stiffer than the CS crosslinked scaffold (data not shown).
4. Discussion

The attachment of CS molecules to silk fibers via covalent cross-linking with free amino group is typically a daunting task, due to the poor availability of pendant amino groups. Mulberry silk, produced by *Bombyx mori*, has an almost negligible free amino group content. The advantage of using a non-mulberry silk, such as that of *Antherea mylitta*, over a mulberry silk is the presence of RGD sequences [44], which offer a significantly greater amount of free amino acid-containing residues (around 5% arginine, as well as some histidine and lysine) [44], thus enabling this variety of silk to undergo chemical modification, e.g. by crosslinking of bioactive ligands.

Adjacent hydroxyl groups on the CS backbone were first converted into aldehyde functional groups by reaction with sodium periodate, and then the intermolecular condensation reaction was carried out between the aldehyde groups of oxidized CS and the amine groups of silk fibroin, forming a Schiff’s base (Fig. 2A and B). After the crosslinking reaction, crosslinked silk fibers turned a deep brown color (Fig. 5A and B), indicating the development of conjugated chemical moieties.

MTT staining showed the presence of metabolically active cells in both types of scaffolds, but were visibly not homogeneously distributed. The seeding efficiency and cell distribution uniformity in fibrous silk scaffolds would be expected to improve by using dynamic conditions in spinner flasks or perfusion-based bioreactors [45].

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The fact that nasal chondrocytes cultured over the silk fibrous scaffolds for 4 weeks not only uniformly followed silk fiber alignment but also deposited abundant amounts of ECM components is a very interesting finding, compared to previous studies culturing AF or NP cells over spatially patterned films or hydrogels for contact guidance, which were unable to demonstrate evidence of substantial matrix deposition [46].

The effect of CS crosslinking on chondrogenic redifferentiation was not statistically significant, compared to constructs based on silk only. The crosslinking reaction of CS to silk fibroin fiber might have hindered some sulphation motifs that, when available, are typically involved in binding of bioactive signalling motifs to cells. Furthermore, the chondrogenic redifferentiation capacity of expanded nasal chondrocytes was found to be drastically high, which could have masked a slight, if not strikingly potent, positive effect of CS. It should also be noted that the constructs were cultured in the presence of a chondrogenic factor, TGFβ3, which might have hindered any potential benefit brought about by the presence of CS moieties. CS was previously identified by Varghese et al. [23] to support the chondrogenic differentiation of bone marrow stromal cells, while in this study we addressed the chondrogenic redifferentiation of terminally differentiated cells, nasal chondrocytes. Moreover, the biological effects of CS seem to be highly dependent of the environment, since CS crosslinked to type I collagen has been reported to promote osteogenic differentiation of human bone marrow stromal cells [47].

A previous study [17] showed that anisotropy and non-linearity of AF tissue, especially one of 30° angle layers with successive alternating architecture, would generate shear stresses at the interface which could not be replicated by other designs, such as random orientation or parallel fiber orientations in laminated constructs. Koepsell et al. [18] demonstrated the importance of fiber orientation with electrospun polycaprolactone fibrous scaffolds that provided strong guidance to cell orientation and matrix distribution when the fibers were oriented, but without achieving appropriate tensile moduli. However, the mechanical properties of a construct depend not only on the strength and alignment of the collagen fibers and the ECM composition, but also on fiber–matrix interactions [48] at the interfaces constituted by collagen crosslinks, proteoglycans, elastin and collagen–proteoglycan association [49]. The differences in stiffness (1.5- to 2.8-fold) observed
after 4 weeks of culture between constructs based on silk-CS scaffolds and control silk scaffolds might thus be related to the deposition of different types of ECM components and proteoglycans, or to a different organization of the ECM, in a way that still remains to be elucidated. Such analysis should be integrated with the assessment of tensile stresses (circumferential, longitudinal and torsion), which are known to play a crucial role in the function of IVD.

5. Conclusions

In this study, a new design of 3-D silk fibroin scaffold has been engineered whereby fibers are oriented at alternating angles in successive layers of a multilayered lamellar architecture. In vitro culture of human nasal chondrocytes on these aligned, concentrically lamellar silk scaffolds allowed the engineering of a tissue in which cells were aligned along silk fibers and which showed...
Fig. 7. TEM images of constructs based on silk-CS scaffolds after 4 weeks of culture. (A and B) ECM proteins produced by the cells follow the same orientation as that of the oval shaped chondrocytic cell. (C) Scattered ECM fibers are self-assembled. (D) Rough endoplasmic reticulum indicates ECM proteins synthesis.

Fig. 8. (A) Macroscopic picture of a construct based on silk fibroin scaffold cultured for 4 weeks in chondrogenic medium. (B) GAG/DNA ratios of constructs based on control or silk-CS scaffolds cultured for 1 day or 4 weeks. (C) Amounts of type II collagen or total collagen (expressed per DNA) in constructs based on control or silk-CS scaffolds and cultured for 4 weeks.
deposition of an oriented cartilaginous matrix, resembling the organization of native annulus fibrosus. Modulation of the surface chemistry of the scaffold by covalent binding of CS did not significantly change the extent of chondrogenic redifferentiation, but it did contribute to the development of a biocomposite construct with similar resilience to annulus fibrosus in native IVD under compression.

This custom-made aligned silk fibrous scaffold with a multilayered lamellar architecture could be used in combination with cells for simulating the precise hierarchical architecture, typical

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**Fig. 9.** Sections of constructs based on control (A, C, E, G, H) or silk-CS scaffolds (B, D, F) after 4 weeks of culture stained by Safranin-O (A, B: constructs generated with cells from donor A), or labeled by immunohistochemistry for collagen type I (C, D, G, H) or collagen type II (E and F) (constructs generated with cells from donor C). The asterisk indicates a cross-section of fibroin fibers; the arrows indicate longitudinal sections of fibroin fibers. Scale bars: A, B, C, D: 200 μm; G: 100 μm; H: 20 μm.
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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1, 2, 4–10, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2012.05.023.

References