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Modulation of extracellular matrix by annulus fibrosus cells on tailored silk based angle-ply intervertebral disc construct

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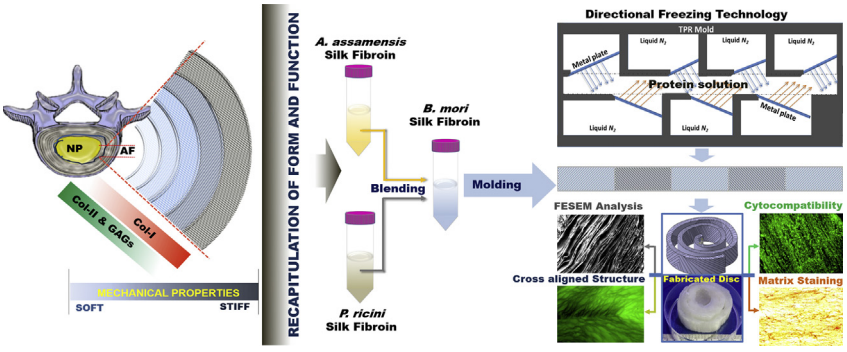
HIGHLIGHTS

- Fabricated silk based full thickness angle-ply construct mimic native intervertebral disc intricacy.
- Blends of two silk fibroin varieties provides differential physicochemical properties in developed constructs.
- The fabricated disc supports proliferation, alignment and maturation of primary annulus fibrosus cells of porcine origin.
- Extracellular matrix turnover is guided by differential mechanical property of fabricated disc.

GRAPHICAL ABSTRACT



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ABSTRACT

Reconstruction of native tissue's anatomical and biophysical milieu dictates the success of tissue engineered graft's cellular fate. Herein, we report a facile fabrication procedure to replicate the anatomical and biomechanical features of annulus fibrosus (AF) tissue. A seamless, full thickness disc-like angle-ply construct was fabricated using silk fibroin (SF) protein. To mimic the gradual transition of mechanical gradient from inner to outer region of native AF tissue, SF proteins from two different sources (namely *Bombyx mori*, BM SF as mulberry, and *Antheraea assamensis*, AA SF and *Philosamia ricini*, PR SF as non-mulberry) were blended that provided differential mechanical and cell binding properties. Fabricated constructs were physicochemically and biologically characterized. The seeded porcine AF cells were found to proliferate and align along the lamellar pores as visualized through staining. Gene expression study concluded higher expression of collagen-I with enhancement of mechanical properties, whereas an opposite trend was observed for both collagen-II and aggrecan. Overall, the angle-ply construct with tailored mechanical properties supported cellular alignment and proliferation, and modulated the extracellular matrix (ECM) deposition forming a functional AF tissue like construct, thus providing a robust foundation as an alternative tissue engineered strategy in intervertebral disc (IVD) regeneration for future replacement therapy.

1. Introduction

Intervertebral disc degeneration (IDD) is one of the prime causes of disability and has significant consequences in socioeconomic altercations implicating patient's quality of health [1]. Intervertebral disc (IVD) consists of two morphologically and developmentally distinct regions; the multi-lamellar hierarchically arranged collagen fiber reinforced annulus fibrosus (AF) that confines the gelatinous nucleus pulposus (NP), together maintaining the intra-discal pressure upon loading [2]. Any damage to AF caused by trauma, or pathophysiological conditions (genetic disposition, matrix modulation by nutrition

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deficiency and aging) eventually develops tears through which gelatinous NP comes out. This results in decrease of the disc height and uneven pressure distribution that finally lead to disc herniation causing low back pain (LBP). The conventional clinical interventions to IDD include pharmacological and physiotherapeutic approaches that relieve early symptoms. Surgical intervention *e.g.,* spinal fusion, disc removal or prosthetic total disc replacement (TDR) are eventually required in the next stage [3]. However, recent studies have demonstrated these procedures alter or restrict the normal spine biomechanics leading to disintegration in adjacent segments and reoperation in patients [4,5]. In this milieu, tissue engineering approaches provide a promising strategy to address disc degeneration by using bioengineered disc that may recapitulate the form and function of native disc.

AF is intrinsically complex tissue that consists of 20–25 concentric rings surrounding the NP. These rings are mainly composed of collagen nano-fibers oriented at the angle varies from +/− 30°–60° (from the central NP outward to the edge of the disc) against its vertebral axis, but in successive layers forming a criss-cross structure [6]. The biochemical and biomechanical properties remarkably alter along the radial direction. The proportion and the organization of extracellular matrix (ECM) components vary with the spatial position in AF. For instance, concentration of type I collagen increases towards the outer AF layers, whereas type II collagen increases towards center. Similarly, aggrecan content is higher in the inner layers of AF (11–20%) than the outer layers (5–8%) [7]. Such variations in ECM composition are maintained by the specific cell types that reside in that spatial region and their associated cellular activities in the corresponding zone possessing different biophysical environments. This regional distinction in cellular function and ECM composition play a vital role on biomechanics of the discs. For example, the outer layers of AF resist the tensile stresses, whereas compressive loads are compensated by inner AF [8].

Static tension of different magnitudes (*i.e.,* the physico-mechanical cues of the substrate) acts as an important modulator for AF cell behavior [9]. Previous studies showed the influence of substrate stiffness on morphology and ECM gene expression of AF cells which are known to be "mechanosensitive cells" [10]. It was reported that collagen type II and aggrecan gene expression increase as the substrate stiffness decreases. Conversely, collagen type I expression increases on stiffer surfaces [9]. It is very important to choose the appropriate substrate mechanical properties for AF tissue engineering. So, an ideal tissue engineered construct should be designed in such way that it not only mimics the anatomical criss-cross structure, but also recapitulates the biomechanical, biochemical and cellular activities of the native AF. To materialize this, our recent efforts are towards approximating the anatomical features to their biomechanical properties for a successful physiologically functional engineered disc.

Recently, numerous approaches have been attempted to recapitulate the form and function of AF tissue, but with limited success. This is a greater need for a strategy to mimic such complex tissue organization at the biochemical and biomechanical levels. For instance, a developed fibrous scaffold of alginate/chitosan supported the canine AF cell growth and specific ECM deposition, however failed to mimic the architecture of AF anatomy [11]. Similarly, biphasic scaffolds made of demineralized bone matrix gelatin (BMG) poly(polycaprolactonetriol malate) (PPCLM) supported the growth of chondrocytes and was biochemically and biomechanically close to native rabbit AF, but failed to mimic the intricate microarchitecture of AF [12]. A recent advancement in these approaches was the use of silk fibers/chondroitin sulfate modified silk fibers to prepare a biochemically gradient criss-cross structure resembling native AF [13,14]. However, there were limitations in the technique *i.e.,* failure to mimic individual cross aligned layer to its vertical axis, porosity limitations hindering cellular migration and breaking of fibers or unwinding issues raising concerns. Moreover, the degradation rate of natural raw silk fibers is very slow, limiting replacement by neotissue under *in vivo* conditions [15]. One very promising approach to address the complex angle-ply architecture of AF was the multi-scale biologic construct using electrospun mat of PCL (polycaprolactone)/poly(ethylene oxide) (PEO) [16–18]. The group focused on transition of engineered disc from controlled *in vitro* condition to less favorable *in vivo* environment, and also on implant integration and remodeling. However, introduction of lamellar scaffolds also provide new insights into AF tissue engineering [19,20].

Silk as biomaterial is used in tissue engineering for decades due to its widespread versatility *e.g.,* biocompatibility, least immunogenicity, tunable biodegradability and mechanical properties [21]. Silk fibroin (SF) from *Bombyx mori* Linnaeus, 1758 (BM SF) is being used in tissue engineering for years [22–26]. Similarly, non-mulberry SF from *Antheraea mylitta* Drury, 1773 (AM SF) has also been extensively studied [27]. Here we studied two other non-mulberry SF sources; *Antheraea assamensis* Helfer, 1837 (AA SF) and *Philosamia ricini* Donovan, 1798 (PR SF). It is reported that non-mulberry silk fibroin possess inherent RGD-motif (arginine-glycine-aspartic acid, an integrin binding receptor) facilitating enhanced cell attachment and proliferation [28,29]. Moreover, the unique molecular architecture (uninterrupted poly-alanine stretches) of AA SF or PR SF provides superior mechanical properties [29]. Recently, few reports suggest the potential use of non-mulberry silk (*e.g.,* AM SF, AA SF and PR SF) in various field of tissue engineering including skin, cartilage, bone, blood vessel and heart [30–35].

In the present study, we developed and applied a novel design to fabricate a seamless lamellar sheet possessing aligned pores at an angle of approximately ±30° to its length, but in alternate directions akin to native AF. Further through this study we thoroughly investigated the effects of structural organization and mechanical properties on extracellular matrix secretion and specific gene expression using our developed silk AF model that provided differential mechanical properties. The effect of substrate morphology and its bulk mechanical properties on growth, expression, and biochemical characteristics of AF cells were also investigated.

2. Materials and methods

2.1. Isolation of mulberry silk fibroin

Mulberry silk fibroin (SF) solution was isolated from *B. mori* cocoons following the procedure described by Kim et al. [36]. In brief, *B. mori* (BM) cocoons were cut into small pieces followed by boiling for 15 min in 0.02 M Na_2CO_3 (Sigma, U.S.A.) solution to remove the glue like sericin from outer layers and then rinsed thoroughly with deionized water. The extracted silk fibers were then allowed to dry for overnight at room temperature (25 °C). The sericin free dry silk fibers were dissolved in 9.3 M LiBr (Sigma, U.S.A.) solution and kept it for 4 h at 60 °C. The liquefied silk was then extensively dialyzed against deionized water using 12 kDa molecular weight cut-off (MWCO) cellulose dialysis membrane (Sigma, U.S.A.) to remove the residual LiBr. Post dialysis, 1 mL of regenerated aqueous BM SF solution was transferred to a teflon-boat of known weight (W1) and was left to dry at 37 °C for 12–16 h. Once silk was dry, weight was measured as W2. The final concentration of the SF solution was 9–12% (w/v), determined gravimetrically using an electronic balance (Sartorius, BSA224S-CW) following the equation expressed as.

$$\%(\mathrm{w/v}) \text{ of SF} = [(\mathrm{W2}-\mathrm{W1})/1\ \mathrm{mL}] \times 100$$

2.2. Isolation of non-mulberry silk fibroin

Aqueous solution of non-mulberry SF was isolated from matured 5th instar larvae of both muga (*A. assamensis,* AA) and eri silkworm (*P. ricini,* PR), respectively following the previously described protocol [37]. Briefly, 5th instar larvae were collected from local sericulture farms of Assam, India. The silk glands of the larvae were taken out and washed with deionized water to remove the traces of water soluble sericin.