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Potential of 3-D tissue constructs engineered from bovine chondrocytes/silk fibroin-chitosan for *in vitro* cartilage tissue engineering

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ABSTRACT

The use of cell-scaffold constructs is a promising tissue engineering approach to repair cartilage defects and to study cartilaginous tissue formation. In this study, silk fibroin/chitosan blended scaffolds were fabricated and studied for cartilage tissue engineering. Silk fibroin served as a substrate for cell adhesion and proliferation while chitosan has a structure similar to that of glycosaminoglycans, and shows promise for cartilage repair. We compared the formation of cartilaginous tissue in silk fibroin/chitosan blended scaffolds seeded with bovine chondrocytes and cultured *in vitro* for 2 weeks. The constructs were analyzed for cell viability, histology, extracellular matrix components glycosaminoglycan and collagen types I and II, and biomechanical properties. Silk fibroin/chitosan scaffolds supported cell attachment and growth, and chondrogenic phenotype as indicated by Alcian Blue histochemistry and relative expression of type II versus type I collagen. Glycosaminoglycan and collagen accumulated in all the scaffolds and was highest in the silk fibroin/chitosan (1:1) blended scaffolds. Static and dynamic stiffness at high frequencies was higher in cell-seeded constructs than non-seeded controls. The results suggest that silk/chitosan scaffolds may be a useful alternative to synthetic cell scaffolds for cartilage tissue engineering.

1. Introduction

Damaged adult articular cartilage exhibits a limited propensity for self-repair. Normal cartilage is composed of chondrocytes in a hydrated extracellular matrix (ECM). The chondrocytes synthesize sulfated glycosaminoglycan (GAG) and type II collagen, which provide the tissue with load-bearing function [1,2]. Current methods of cartilage repair, including microfracture, osteochondral grafts, and prosthetic joint replacement do not provide for long-lasting and complete recovery [3–7]. Thus, engineered tissue implants are attractive and offer a promising approach to cartilage restoration.

Formation of cartilaginous constructs *in vitro* involves manipulation of four parameters: scaffold, cells, soluble factors, and the physical environment [8]. A variety of biomaterials, both natural and synthetic, have been analyzed to form scaffolds and tested for cartilage tissue engineering (Table 1). Physiologic biomaterials include fibrin, hyaluronic acid, and various forms of collagen. Natural materials include alginate, chitosan, and silk fibroin. Synthetic materials include poly (glycolic acid) (PGA) and poly (lactic acid) (PLA). A number of natural and synthetic materials undergo relatively rapid degradation, during which their size, shape, and function changes [9]. The effects of this degradation on the formed construct may include physical and chemical, such as due to formation of acid by-products [10]. In contrast, more stable scaffold or hydrogel materials, such as agarose [11], allow for analysis of the contribution of cells and matrix deposited in the material.

Silk fibroin (SF) is an attractive natural fibrous protein for biomedical applications and studies due to a number of biological, chemical, and physical properties. SF facilitates cell adhesion and growth, and has relatively low thrombogenicity, low inflammatory

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Table 1
List of some representative biomaterials (natural and synthetic) used for different chondrocytes based cartilage tissue engineering.

Materials	Cell types	Methods	Findings	References
Silk fibroin	Rabbit chondrocytes	SEM, Safranin O staining, immunohistochemical staining	Comparison of salt leached and solvent based scaffolds	[43]
Silk fibroin	Human chondrocytes	Histology, RTPCR, Alcian Blue staining	Comparison of 2-D and 3-D matrices	[17]
Chitosan	Porcine chondrocytes	Quantitative analysis, SEM	Effect of varying pore size	[31]
Chitosan	Sheep chondrocytes	Biochemical and biomechanical analysis, histology, *in vivo* studies	*In vivo* cartilage repair in sheep	[44]
Chitosan/polyester	Bovine chondrocytes	Microcomputed tomography, histology	Effect of pore size and geometry of pore	[7]
Silk fibroin/chitosan	Bovine chondrocytes	Biochemical and biomechanical analysis, Histology, SEM, Immunohistochemistry	Effect of different chemical composition	This study
Collagen	Porcine chondrocytes	SEM, cell viability, Histology, RTPCR	Effect of different matrices	[47]
Gelatin	Rat chondrocytes	Massons stain, SEM	Effect of novel gel cross-liking method	[48]
Gelatin–chondroitin–hyaluronan	Porcine chondrocytes	SEM, histology	Comparison of static and dynamic culture conditions	[49]
Chondroitin-6-sulfate/dermatan sulfate/chitosan	Rat chondrocytes	RTPCR, immuno histochemical staining	Response surface methodology used for scaffolds preparation	[50]
Poly (glycolic acid)	Porcine chondrocytes	Cryopreservation, DNA content determination	Effect of cryopreservation	[51]
Poly (€-caprolactone)	Porcine chondrocytes	SEM, histology, qtPCR	Effect of material design on chondrogenesis	[52]

response, and low protease susceptibility when highly crystallized [12–15]. Furthermore, silk can be subjected to aqueous or organic solvent processing and can be chemically modified to address a wide range of applications. SF scaffolds can provide high permeability to oxygen and water as well as robust mechanical properties. Recent cartilage tissue engineering studies performed using silk scaffolds resulted in adhesion and proliferation of chondrocytes and mesenchymal stem cells, and production of cartilaginous matrix *in vitro* [16,17]. Thus, SF is attractive for studies of cartilage tissue engineering, and because of its slow degradation, SF may be blended with other materials to form suitable scaffolds.

Chitosan (CS) is a biomaterial that mimics the glycosaminoglycan (GAG) components of cartilage. CS is a partially deacetylated derivative of chitin found in arthropod exoskeletons. It consists primarily of repeating units of β (1–4) linked glucosamine and N-acetyl glucosamine. It is formed through the N-deacetylation of chitin and structurally similar to GAGs. Chitosan supports chondrogenic activities [18–25] and is being evaluated in cartilage tissue engineering applications. Chitosan has also roles in wound healing, is non-toxic, and generates a minimal foreign body response with accelerated angiogenesis [26]. The properties of porous chitosan matrices such as microstructure, crystallinity, and mechanical strength can be varied by altering chitosan concentration, freezing rate, the molecular weight and percent deacetylation [9,27–29]. Despite the growing interest for chitosan as a biomaterial for tissue engineering, most studies on pure chitosan scaffolds have focused on sponges [8,30–36] or hydrogels [20,37]. Porous scaffolds allow seeding of cells with desirable and tunable characteristics such as biocompatibility, mechanical properties and biodegradability [12–15,38,39].

Silk-chitosan blend hybrid material may have beneficial properties, as shown for the culture of HepG2 hepatocyte and fibroblast cells [40–42]. Although silk fibroin and chitosan have been studied separately for *in vitro* chondrogenesis [17,31,43,44], the influence of silk fibroin/chitosan composite scaffolds on chondrocyte morphology, differentiation, and function has not been studied yet and no study of this type has been performed earlier on chondrocytes. Earlier, we fabricated and characterized the polyelectrolyte complex porous scaffolds of silk fibroin/chitosan and investigated their suitability for tissue engineering applications [42]. Silk fibroin and silk fibroin/chitosan blended scaffolds of different ratios (1:1 and 2:1) appeared promising based on cell viability and attachment. Thus, these scaffolds are used in the present study to evaluate the silk fibroin/chitosan blended scaffolds as matrices using bovine chondrocytes to analyze the cellular activity, viability, biochemical and biomechanical properties for cartilage tissue engineering.

2. Materials and methods

2.1. Materials

For scaffolds, CS derived from crab shells with a deacetylation degree of >85% was purchased from Sigma Aldrich (St. Louis, MO USA), and silk cocoons were kindly provided by Debra silkworm farm (West Bengal, India). For chondrocyte isolation and culture, biochemical, and immunochemical analyses, reagents were obtained as described previously [45,46].

2.2. Experimental design

The study design is summarized in Fig. 1. Porous scaffolds of (1) SF alone, and SF blended with CS at two ratios (2) SF/CS (1:1), and (3) SF/CS (2:1) were compared for their ability to support the formation of cartilaginous tissues. Scaffolds were either analyzed after preparation (a) directly (without seeded cells) or (b) with seeded chondrocytes and two weeks of incubation. Some samples were analyzed for cartilaginous matrix components, sulfated glycosaminoglycan (GAG) and collagen (COL) while others were analyzed for viable vs. non-viable cells by staining with fluorescent indicators, for the location of GAG by histochemical staining, for the location of types I and II collagen by immunohistochemistry, and for compressive load-bearing properties by static and dynamic compression testing.

2.3. Preparation and characterization of scaffolds

Porous scaffolds of silk fibroin (SF) alone and with blends of chitosan (CS) were fabricated as described previously [42,53]. Briefly, CS was dissolved in 2% acetic acid and clarified by centrifugation. The final concentration of chitosan was 2%. SF solution was prepared following the method of Sofia et al. [53], with slight modification. Briefly, silk cocoons were cut into pieces, degummed (to remove sericin) with a boiling 0.02 M Na_2CO_3 solution for 30 min, and the fibers were washed with elix (deionized) water and then kept at 37 °C overnight to dry. Purified fibers were dissolved in 9.3 M LiBr and then dialyzed against water using a 12 kDa molecular weight cutoff cellulose dialysis membrane. Dialysis was carried out to remove LiBr from the silk fibroin solution. The final concentration of silk fibroin solution used was 2% and was determined gravimetrically by drying the solution. SF, SF/CS (1:1) blend, and SF/CS (2:1) blends were prepared and used to fabricate scaffolds by freezing the solution at −20 °C and then lyophilizing for 36 h. Scaffolds were then treated with a gradation of ethanol (100% ethanol for 1 h, 70% for 30 min, and 50% ethanol for 30 min) to neutralize and sterilize the scaffolds [42,54]. Some scaffolds were characterized for pore structure by sputter-coating with gold, viewing by scanning electron microscopy (SEM) with a JEOL-JSM 5800 SEM, and recording images. Pore size was then determined by measuring pore diameter in >30 pores using Image J software (Wayne Rasband, National Institute of Health, USA). Before use in tissue engineering experiments, scaffolds were cut to ~2 mm thickness, punched to 6.4 mm diameter, and rinsed with culture medium as described below.

2.4. Isolation of bovine chondrocytes

Chondrocytes were isolated from the knees of immature bovine calves by sequential enzymatic digestion [44,55]. Two preparations of cells were generated,