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Silk microfiber-reinforced silk hydrogel composites for functional cartilage tissue repair

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ABSTRACT

Cartilage tissue lacks an intrinsic capacity for self-regeneration due to slow matrix turnover, a limited supply of mature chondrocytes and insufficient vasculature. Although cartilage tissue engineering has achieved some success using agarose as a scaffolding material, major challenges of agarose-based cartilage repair, including non-degradability, poor tissue–scaffold integration and limited processing capability, have prompted the search for an alternative biomaterial. In this study, silk fiber–hydrogel composites (SF–silk hydrogels) made from silk microfibers and silk hydrogels were investigated for their potential use as a support material for engineered cartilage. We demonstrated the use of 100% silk-based fiber–hydrogel composite scaffolds for the development of cartilage constructs with properties comparable to those made with agarose. Cartilage constructs with an equilibrium modulus in the native tissue range were fabricated by mimicking the collagen fiber and proteoglycan composite architecture of native cartilage using biocompatible, biodegradable silk fibroin from *Bombyx mori*. Excellent chondrocyte response was observed on SF–silk hydrogels, and fiber reinforcement resulted in the development of more mechanically robust constructs after 42 days in culture compared to silk hydrogels alone. Thus, we demonstrate the versatility of silk fibroin as a composite scaffolding material for use in cartilage tissue repair to create functional cartilage constructs that overcome the limitations of agarose biomaterials, and provide a much-needed alternative to the agarose standard.

1. Introduction

Epidemiological studies have revealed that more than 70% of adults between the ages of 55 and 78 experience disability due to osteoarthritis [1]. Coupled with high disease prevalence, the limited capacity of adult cartilage to undergo self-regeneration has prompted the urgent development of functional cartilage tissue replacement therapy. Poor vascularization, slow matrix turnover, a limited number of progenitor cells and a scarcity of mature, non-dividing chondrocytes all contribute to the inability of cartilage lesions to heal, particular in the elderly [2]. While autografting, allografting and microfracturing used currently to surgically repair lesions have provided some relief, these procedures are not linked with high success rates [3].

Cartilage tissue engineering (TE) has emerged as a promising therapeutic for joint repair. Since native cartilage demonstrates compressive moduli of the order of 300–800 kPa [4], mechanical strength and structural resilience is one of the key requirements for cartilage scaffold fabrication [5]. At a structural level, native cartilage is a multilayer connective tissue composed of chondrocytes dispersed in a dense extracellular matrix (ECM). The hydrophilic environment of the ECM, due to negatively charged glycosaminoglycans (GAGs) that attract water, enables cartilage tissue to experience a swelling pressure, which is countered by the tensile strength generated from the interspersed collagen network [6]. Despite modest success in cartilage TE, the non-homogeneous, depth-dependent composition of articular cartilage, coupled with differences in layer thickness, cellular morphology and ECM composition, makes this tissue difficult to mimic structurally [7–9].

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Unlike other scaffold formats, hydrogels provide swelling kinetics and a hydrated environment similar to native tissue [5]. The injectability of hydrogels into a damaged site, the shape-conforming capacity of the gel within the defect, and the ability to homogeneously suspend cells within the hydrogel network and preserve chondrogenic phenotype in vitro makes hydrogels a highly attractive platform for cartilage repair [10,11]. The development of functional cartilage tissue has evolved through the use of various synthetic and natural hydrogel materials. While synthetic polymers, such as poly(ethylene glycol) (PEG) or polyglycolic acid (PGA), provide well-controlled systems for determining the effects of isolated material properties on scaffold design (e.g. degree of crosslinking, mechanics), these materials are relatively inert and offer limited support for chondrogenesis and cartilage matrix production [12,13]. Instead, natural materials, such as collagen, hyaluronic acid, agarose, alginate, fibrin and elastin, are favored due to their abundance, environmentally friendly processing and inherent prochondrogenic properties [14].

Agarose hydrogels in particular have been studied extensively to determine the effects of factors such as mechanical loading and cell-seeding density on cartilage tissue formation [15]. Due to its superior support for chondrogenesis and higher deposition of GAG compared to other natural hydrogels made from fibrin, collagen, alginate and PGA, agarose has been labeled as the "gold standard" biomaterial for in vitro cartilage tissue formation [16]. However, despite its promising performance in vitro, agarose has undergone limited study in animal models due to its poor biocompatibility and inability to degrade in vivo, which prevents graft integration with the host tissue [17]. Furthermore, cartilage tissue formation within an agarose hydrogel is complicated by an inability to customize agarose scaffold structure and composition [18,19]. Thus, limitations of agarose-based joint therapy have prompted the search for an alternative natural biomaterial for cartilage tissue engineering.

Silk fibroin from *Bombyx mori* silkworms is a promising substitute for agarose in cartilage repair due to its robust mechanical properties, superior biocompatibility, degradability, ease of fabrication and tunable processing parameters [20]. Extensive in vivo study on the implantation of silk scaffolds has proven that silk elicits little to no immune response, degrades in a controlled manner via ubiquitous protease-mediated digestion, and can be conjugated with functional groups or RGD peptide modification to promote cell adhesion [21–23]. In addition, silk fibroin can easily be assembled into a versatile array of material formats (e.g. films, sponges, microspheres, electrospun fibers, hydrogels) using aqueous-based processing [24]. The formation of silk hydrogels in particular is accomplished by a variety of mechanisms marked by a change in silk conformation from amorphous random coil to organized crystalline β-sheet structures. The silk sol–gel process that leads to physical alignment of the protein chains relies on a balance between protein concentration, temperature, pH and salt/ion concentration during the phase transition [25]. Sonication-mediated gelation utilizes shear force from ultrasound waves to initiate gelation, and affords fine control over gelation kinetics by tuning silk fibroin concentration or sonication parameters, such as duration time and energy output. Thus, this time delay allows for encapsulation of cells or biomolecules within the silk prior to gelation [26].

Previously, we demonstrated that one type of silk hydrogel, prepared by sonication-induced gelation of a silk solution, could support chondrocyte viability and yield cartilaginous constructs with biochemical properties mimicking those of native cartilage tissue [27]. While these silk hydrogels provided an excellent scaffold for chondrocyte attachment and cartilage matrix deposition, further improvement in the mechanical properties of these hydrogels is necessary to construct optimal load-bearing cartilage tissue constructs. Efforts in cartilage TE over the past few decades have improved hydrogel mechanics using methods such as chemical crosslinking [28,29], double-network hydrogels [30,31] and hydrogel interpenetrating scaffolds [32,33]. While fiber reinforcement has also successfully enhanced the mechanical performance of hydrogel systems, little is known about the effects of fiber–gel composite systems on long-term cell viability and tissue development [34,35].

The aim of this study was to leverage the versatility of silk fibroin to generate a mechanically reinforced silk microfiber silk hydrogel (SF–silk hydrogel) composite for functional cartilage TE. Osteoconductive materials have been successfully developed using silk microfiber-reinforced porous scaffolds; however, silk fiber reinforcement has not yet been applied to a hydrogel system [36]. For the first time, two formats of silk—microfiber and sonicated hydrogel—have been united to develop a mechanically reinforced hydrogel for cartilage tissue engineering. The composite SF-silk hydrogel system was optimized according to diffusivity and mechanical properties, and compared to the agarose hydrogel standard to identify a potential alternative to the current "gold standard" biomaterial. Silk microfibers and primary chondrocytes were encapsulated within the gel during the sonication-induced sol–gel transition to test the hypothesis that SF–silk hydrogels exhibit comparable properties to agarose and can yield cartilage constructs that mimic native cartilage after only 6 weeks of in vitro culture. Through this work, we provide valuable insights into the role of silk microfibers in hydrogel reinforcement, and demonstrate the development of SF–silk hydrogel cartilage constructs with properties approaching those of native cartilage.

2. Materials and methods

2.1. Preparation of silk solution

Silk fibroin was extracted from cocoons of *B. mori* as previously described [27]. Briefly, silk cocoons were boiled in an aqueous solution of 0.02 M Na_2CO_3 and washed with deionized (DI) water. The resultant dry silk fibroin was then dissolved in a 9.3 M LiBr solution (25% w/v) at 60 °C for 4–6 h, and dialyzed against DI water using 3500 dalton molecular weight cut off dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA). The final concentration of the aqueous silk solution was 6–8% w/v, which was determined by weighing the remaining silk solid after drying a known volume.

2.2. Preparation of silk microfibers

Micron-sized, non-immunogenic silk fibers were fabricated according to protocol by Mandal et al. [36]. Briefly, 0.35 g of dried, degummed silk fibers were incubated in a 17.5 M NaOH solution. To obtain large (>500 μm), medium (400–500 μm) and small (150–200 μm) microfibers, the hydrolysis reaction was carried out for 30, 60 and 180 s, respectively. The reaction was quenched with DI water and the microfibers were washed repeatedly. Dried fibers were subsequently obtained through lyophilization. Microfibers were stored at ambient conditions until further use.

2.3. Chondrocyte isolation

Cartilage was harvested from fresh bovine carpometacarpal joints of 4 month old calves (Green Village Packing Co., NJ). Cartilage flakes were digested for 10 h using collagenase (390 U ml^{-1}, type V; Sigma Aldrich, St Louis, MO) in high-glucose Dulbecco's Modified Eagle's Medium (hgDMEM supplemented with 5% FBS). The digesting suspension was filtered through a 70 μm cell

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