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Silk fibroin-chondroitin sulfate scaffold with immuno-inhibition property for articular cartilage repair

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ABSTRACT

The demand of favorable scaffolds has increased for the emerging cartilage tissue engineering. Chondroitin sulfate (CS) and silk fibroin have been investigated and reported with safety and excellent biocompatibility as tissue engineering scaffolds. However, the rapid degradation rate of pure CS scaffolds presents a challenge to effectively recreate neo-tissue similar to natural articular cartilage. Meanwhile the silk fibroin is well used as a structural constituent material because its remarkable mechanical properties, long-lasting *in vivo* stability and hypoimmunity. The application of composite silk fibroin and CS scaffolds for joint cartilage repair has not been well studied. Here we report that the combination of silk fibroin and CS could synergistically promote articular cartilage defect repair. The silk fibroin (silk) and silk fibroin/CS (silk-CS) scaffolds were fabricated with salt-leaching, freeze-drying and crosslinking methodologies. The biocompatibility of the scaffolds was investigated *in vitro* by cell adhesion, proliferation and migration with human articular chondrocytes. We found that silk-CS scaffold maintained better chondrocyte phenotype than silk scaffold; moreover, the silk-CS scaffolds reduced chondrocyte inflammatory response that was induced by interleukin (IL)-1β, which is in consistent with the well-documented anti-inflammatory activities of CS. The *in vivo* cartilage repair was evaluated with a rabbit osteochondral defect model. Silk-CS scaffold induced more neo-tissue formation and better structural restoration than silk scaffold after 6 and 12 weeks of implantation in ICRS histological evaluations. In conclusion, we have developed a silk fibroin/ chondroitin sulfate scaffold for cartilage tissue engineering that exhibits immuno-inhibition property and can improve the self-repair capacity of cartilage.

Statement of Significance

Severe cartilage defect such as osteoarthritis (OA) is difficult to self-repair because of its avascular, aneural and alymphatic nature. Current scaffolds often focus on providing sufficient mechanical support or bio-mimetic structure to promote cartilage repair. Thus, silk has been adopted and investigated broadly. However, inflammation is one of the most important factors in OA. But few scaffolds for cartilage repair reported anti-inflammation property. Meanwhile, chondroitin sulfate (CS) is a glycosaminoglycan present in the natural cartilage ECM, and has exhibited a number of useful biological properties including anti-inflammatory activity. Thus, we designed this silk-CS scaffold and proved that this scaffold exhibited good anti-inflammatory effects both *in vitro* and *in vivo*, promoted the repair of articular cartilage defect in animal model.

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1. Introduction

Articular cartilage is the thin layer tissue that protects the subchondral bone from high stress by providing efficient load

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distributions through its specific extracellular matrix structure such as collagen and chondroitin sulfate. Severe cartilage damage due to developmental abnormalities, trauma, injuries or aging-related degeneration is difficult to self-repair because of its avascular, aneural, and alymphatic nature, and this could lead to the progressively degenerative disease, osteoarthritis (OA) [1]. Increasing number of people world-wide is suffering from the joint-related diseases caused chronic pain and mobility loss, which is severely affecting the quality of life and work productivity. Currently, several surgical methods have been employed to treat cartilage defects, including microfracture, osteochondral grafts and prosthetic joint replacement, etc. [2,3]. However, the shortage of donor tissues, requirement of a secondary surgery, and possible morbidity have limited the application of these cartilage repair techniques [4]. Alternatively, cartilage tissue engineering, which can repair cartilage defects with minimally invasive operation, becomes a promising approach.

The emergence of cartilage tissue engineering has increased the demand for scaffolds capable of providing a favorable environment for mechanical support, chondrogenic cell growth and new cartilage-specific extracellular matrix (ECM) formation [5–8]. Synthetic and natural polymers are primarily used to create the three dimensional environment. Although synthetic polymers are easy to manufacture and duplicate, natural polymers possess intrinsic advantages that can be exploited. Collagen [2,9,10], fibrin [11,12], hyaluronic acid [13,14], alginate [15,16], chitosan [17,18], silk fibroin [19–21], agarose [22,23] and other natural materials [24,25] have been used for cartilage tissue engineering applications and achieved certain results. Silk fibroin (SF) is an attractive natural fibrous scaffold material among these natural polymers, because it offers versatile processability, slow biodegradation, excellent biocompatibility and robust mechanical properties. Furthermore, SF provides ideal bioactivity as a tissue-engineering scaffold – it facilitates cell adhesion and growth, and has relatively low levels of thrombogenicity, inflammatory response [26], and protease susceptibility when highly crystallized. SF is widely used in the form of films, membranes, gels, sponges, powders, and scaffolds [27,28]. We have previously applied silk fibroin scaffold for tendon and cartilage regeneration with satisfactory results [29–31].

Chondroitin sulfate (CS) is a glycosaminoglycan (GAG) presented in the natural cartilage ECM, and it maintains the structural integrity of cartilage and helps to restore arthritic joint function through a number of useful biological properties such as anti-inflammatory activity [32], stem cell niche maintenance [33] and regulating enzymatic activity [34]. Scaffolds made from CS have been widely applied in nucleus pulposus [35], corneal stromal [36], tendon [37], bone [38], and cartilage [39] tissue engineering. However, rapid degradation rate of pure CS scaffolds presents a challenge to effectively recreate neo-tissue similar to natural articular cartilage in diseased cartilage [40], particularly when inflammation became one of the most important factors in the osteoarthritic joints [41–43].

To solve the problems of rapid degradation and less strength of pure CS scaffold, we therefore introduce the silk fibroin into the cartilage tissue-engineering scaffold. In the present study, we have designed and generated a silk-CS scaffold based on salt-leaching, freeze-drying and crosslinking methodologies, and we hypothesized that this silk-CS scaffold could promote the repair of articular cartilage defect with potentially anti-inflammatory effect. The *in vitro* biocompatibility of the scaffolds was investigated by cell adhesion, proliferation and migration with human articular chondrocytes, and the *in vivo* cartilage repair was evaluated with a rabbit osteochondral defect model.

2. Experimental section

2.1. Preparation of silk fibroin solution

Raw *Bombyx mori* silk fibers were purchased from Zhejiang Cathaya International Co., Ltd. *Bombyx mori* silk fibroin was prepared as reported elsewhere with minor modifications [44]. In brief, fibers were boiled in an aqueous sodium carbonate solution (0.02 M) thrice (1 h each time) and rinsed thoroughly with distilled water in order to extract the glue-like protein sericin and wax. The purified silk fibroin was dissolved in 9 M lithium bromide (LiBr) solution at 60 °C for 4 h, yielding a 16% (w/v) solution. The solution was dialyzed in distilled water using a cellulose membrane (molecular weight cut-off: 14,000) for 96 h. Next, the silk fibroin solution was collected and centrifuged at 3500 rpm for 10 min. The aqueous solution was treated at −80 °C overnight, freeze-dried and stored at 4 °C until further use.

2.2. Fabrication of silk and silk-CS scaffold

The prepared silk fibroin was dissolved in distilled water and the aqueous solution was diluted to 6 wt%. Chondroitin sulfate (Sigma) was also dissolved to the silk aqueous solution to get 2 wt% (concentration details in Supplementary Fig. 1). The silk scaffolds or silk-chondroitin sulfate scaffolds were prepared by adding 0.5 g of granular sodium chloride and transferring 0.5 ml of silk fibroin solution or silk-chondroitin sulfate solution into a customer-designed mold (4 mm inner diameter), followed by pushing the silk fibroin solution to the bottom of the mold for scaffold molding. Following this, the scaffold was crosslinked at 4 °C for 36 h, and dried at room temperature for 5 h and then in the oven at 60 °C overnight. In order to extract the sodium chloride, the scaffold was released from the mold and rinsed thoroughly with distilled water. Finally, the scaffolds were obtained by freezing at −80 °C overnight and freeze-drying. The silk-chondroitin sulfated blended scaffolds were cut into 2 mm in height and reacted with 2 wt% chondroitin sulfate solution under 1 wt% genipin solution at 37 °C for 48 h [45]. After that, obtained silk-CS scaffold was washed with PBS to remove the excess crosslinker, air-dried before further use. To prepare the scaffolds for cell culture, silk or silk-CS scaffolds were immersed in 70% ethanol three times (60 min each time), washed with sterile PBS three times and air-dried.

2.3. Scanning electron microscopy (SEM)

To evaluate the microstructure of the scaffold, it was gold-sputtered and observed under a scanning electron microscope (S260 Cambridge, England).

2.4. Swelling ratio of the silk-CS scaffold

Swelling ratio of the silk-CS scaffold is examined according to the methods described previously [46]. Dried scaffolds were immersed in PBS (PH = 7.4) at 37 °C. After 5, 10, 20, 30, 40, 60, 80, 100 and 120 min of immersion, scaffolds were weighted (Ws) with a microbalance after the excess of water on the surfaces was absorbed with filter paper. Swelling ratio (SR) was calculated using the following equation:

$$SR = (Ws - Wd)/Wd. \qquad (1)$$

where Ws and Wd are the weights of the scaffolds at equilibrium swelling state and original state, respectively. Data is presented as mean ± SD, n = 3.

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