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Collagen/silk fibroin composite scaffold incorporated with PLGA microsphere for cartilage repair

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ABSTRACT

For cartilage repair, ideal scaffolds should mimic natural extracellular matrix (ECM) exhibiting excellent characteristics, such as biocompatibility, suitable porosity, and good cell affinity. This study aimed to prepare a collagen/silk fibroin composite scaffold incorporated with poly-lactic-co-glycolic acid (PLGA) microsphere that can be applied in repairing cartilage. To obtain optimum conditions for manufacturing a composite scaffold, a scaffold composed of different collagen-to-silk fibroin ratios was evaluated by determining porosity, water absorption, loss rate in hot water, and cell proliferation. Results suggested that the optimal ratio of collagen and silk fibroin composite scaffold was 7:3. The microstructure and morphological characteristics of the obtained scaffold were also examined through scanning electron microscopy and Fourier transform infrared spectroscopy. The results of in vitro fluorescence staining of bone marrow stromal cells revealed that collagen/silk fibroin composite scaffold enhanced cell proliferation without eliciting side effects. The prepared composite scaffold incorporated with PLGA microsphere was implanted in fully thick articular cartilage defects in rabbits. Collagen/silk fibroin composite scaffold with PLGA microspheres could enhance articular cartilage regeneration and integration between the repaired cartilage and the surrounding cartilage. Therefore, this composite will be a promising material for cartilage repair and regeneration.

1. Introduction

Articular cartilage is an avascular tissue nourished by synovial fluid. However, articular cartilage exhibits poor regenerative capacity after degeneration or injury [1–3]. To resolve this problem, researchers apply tissue engineering as a promising strategy for cartilage regeneration. In this strategy, artificial biomaterials or scaffolds were used to control structural and morphological features of live cells and bioactive molecules [4–6]. To enhance matrix facilitating cartilage repair, researchers should consider ideal scaffolds exhibiting excellent characteristics, such as biocompatibility, suitable porosity, and good cell affinity [7]. Although scaffolds have been developed and used in cartilage regeneration therapy, challenges remain [8].

Collagen (COL), one of the major components of extracellular matrix, displays excellent biocompatibility, negligible immunogenicity, cell adhesion, and biodegradability. COL also specifically interacts with growth factors which promote cell ingrowth and remodeling [9,10]. Scaffolds composed of COL from diverse animal tissues have been actively used in research and clinical applications [11]. Fibrillated collagen matrices in the form of lyophilized hydrogels [12], sponges [13], and films [14] have been investigated. Despite these advantages, the applying of collagen is commonly limited by poor mechanical properties.

Composite materials composed of silk fibroin (SF) and COL may exhibit an effective improvement of characteristics. SF, a protein spun by silkworms, has been widely used as a scaffold material in cartilage tissue engineering [15,16]. As a natural biomaterial, SF is superior in biocompatibility and able to support appropriate cellular activity without eliciting immune activation on hosts [17]. In addition to traditional use as sutures, SF has been exploited as a biomaterial in cell culture and tissue engineering in vitro and in vivo, [18]. Versatile as SF was, this protein can be processed in different formats that satisfy tissue engineering requirements. In this study, a collagen/silk fibroin composite scaffold was developed whose optimum conditions were also investigated in terms of physical properties and biocompatibility to manufacture COL and SF. PLGA-based microspheres encapsulating a transforming growth factor-β1 (rhTGF-β1) were prepared and embedded in COL and SF scaffolds to evaluate the role of scaffold material in controlling the release features of microspheres. Optimal composite-to-PLGA microsphere ratio was then obtained to repair cartilage defect. The developed scaffolds can be applied in repairing cartilage defects.

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2. Materials and methods

2.1. Preparation of materials

SF was produced in accordance with the method described by Zhang et al. [19]. In brief, silkworm cocoons were degummed by boiling in 0.2 M Na_2CO_3 twice for 40 min, following by, rinsing the degummed cocoons thoroughly with water to extract glue-like sericin proteins. The degummed silk fibers were then dissolved in a $CaCl_2/H_2O/CH_3CH_2OH$ solution (molar ratio of 1:8:2) at 80 °C. The resulting mixture was dialyzed in distilled water for 3 d. The concentration of the formed aqueous solution was determined by weighing the residue after the solution was dried.

COL solutions were prepared according to our previously published procedures [20]. In brief, silver carp skins were cut into small pieces, mixed with 1% H_2O_2 in 0.01 M NaOH, and stirred for 24 h. Afterward, the resulting mixture was washed with distilled water until an approximately neutral state was achieved. The processed skins were defatted with 10% isopropyl alcohol, washed, and immersed in 2.5% NaCl for 10 h. After 10 h, the obtained carp skins were extracted by adding pepsin to acetic acid. The solution was centrifuged, and the obtained sediment was dialyzed in 0.1 M acetic acid and distilled water. COL was then lyophilized.

2.2. Analysis of the optimal ratio of composite scaffold

2.2.1. Composite scaffold fabrication

Collagen solution was added to SF solution in weight ratios of 0:10, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 10:0 and then stirred for 3 h. Composite scaffolds were cross-linked with 10 mM ethylene diaminecarbodiimide (EDC) and N-hydroxysuccinimde (NHS) for 8 h. The cross-linked materials were washed with ultrapure water and then lyophilizated.

2.2.2. Water absorption

The lyophilized composite samples (W_1, size = 1 cm × 1 cm) were immersed in PBS for 24 h, dried with filter paper to remove water adsorbed on surfaces, and weighed (W_2). The water content of the composite scaffolds was calculated as follows [13]:

$$\text{Water uptake}\,(\%) = (W_2 - W_1)/W_1 \times 100\%$$

2.2.3. Swelling properties

Scaffold samples (size = 1 cm × 1 cm) were placed in distilled water for 24 h at room temperature and dried in a drying oven. The following equation was used to express percent swelling at equilibrium (Sw) [21]:

$$\text{Sw}\,(\%) = (M_0 - M_1)/M_0 \times 100\%$$

where M_0 refers to the initial weight before the sample is immersed and M_1 refers to the weight after the sample is dried.

2.2.4. Porosity

The porosity of the composite was calculated on the basis of initial ethanol volume (V_1), total volume of scaffold and ethanol (V_2), and residual ethanol volume (V_3) in the containing vessel by using the following equation [22]:

$$\text{Porosity}\,(\%) = (V_1 - V_3)/(V_2 - V_3) \times 100\%.$$

Triplicate measurements were carried out to obtain mean values.

2.2.5. Cell culture

The Bone marrow stromal cells (BMSCs) were isolated from bone marrow of rat tibias and femurs based on MSCs' selectively adherence to plastic surfaces [23]. BMSCs were cultured in high glucose DMEM/F-12 supplemented with 10% FBS as well as antibiotic–antimycotic solution containing 100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin sulfates. After 4 passages, the BMSCs were used for experiments.

2.2.6. Cell proliferation

Cell viability and proliferation on scaffolds were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining. The scaffolds (diameter = 12 mm) were sterilized by γ-rays before placing in culture plates with 1×10^5 cells and incubated at a humidified atmosphere of 5% CO_2, 95% air at 37 °C for 5 h. Scaffolds cultured with BMSCs were then transferred to new culture plates to remove non-adherent cells. Culture medium was changed once at an interval of 2 d. BMSC proliferation was assessed after 1, 3, and 7 days of cell culture. After aspirating culture medium at the predetermined time point, the cell-seeded materials were incubated in MTT solution at 37 °C for 4 h. Dimethyl sulfoxide (DMSO) was mixed in each well to dissolve formazan precipitate. Then, 100 μL of the solution was added to a 96-well plate for the absorbance measurement at 490 nm using a microplate reader. The viability of cells was linearly correlated with optical density (OD). Six parallel replicates (n = 6) were evaluated for each sample and the maximum and minimum were deleted.

2.3. Characterization methods

Scanning electron microscopy (SEM) was performed to observe the morphological characteristics of COL/SF composite scaffolds. Before SEM was conducted, the dried composite scaffolds were cut with a razor blade in liquid nitrogen and then coated with platinum. Fourier transform infrared spectroscopy (FTIR, Spectrum BX, Perkin Elmer, USA) was conducted to observe COL/SF.

2.4. Acridine orange/ethidium bromide (AO/EB) staining

Cell proliferation was determined in accordance with ISO10993-5 standard test method to investigate the relationship of scaffold with cell viability and proliferation [24]. The scaffolds were sterilized by γ-rays and then incubated in DMEM/F12 containing 15% FBS at 37 °C for 48 h at an extraction ratio of 5 mg/mL. The extraction solution was stored at 4 °C for further experiments.

AO/EB double staining was conducted to detect the cell viability of BMSCs in the extraction solution [25]. In brief, BMSCs were harvested and stained with AO/EB solution containing 100 μg/mL AO and 100 μg/mL EB for 15 min, washed twice with PBS, and observed under an Olympus fluorescence microscope.

2.5. Preparation of PLGA microsphere

The microsphere formulations encapsulating TGF-β1 were prepared using a double emulsion–solvent evaporation technique [26]. In brief, 150 μg/mL TGF-β1 was poured in 2.5 mL of a PLGA solution in methylene chloride (250 mg, corresponding to a concentration of 10% w/v). Primary emulsion was generated using a high-speed homogenizer operating at 5000 rpm to 15,000 rpm for 2 min. Afterward, the emulsion was added to 25 mL of 0.5% (w/v) aqueous PVA and homogenized at 8000 rpm for 1 min to produce multiple emulsion. Solvent evaporation and subsequent microsphere hardening were achieved through magnetic stirring at room temperature. After 4 h, microspheres were collected, washed five times with distilled water through centrifugation, and freeze-dried.

2.6. Animal surgery and histological analysis

All of the animals were obtained from the Medical University of Fujian Institutional Animal Care and Use Committee. COL/SF and COL/SF incorporated with TGF-β1 microsphere scaffolds were cut into pieces of Φ 4 mm × 2 mm and used for implantation. Under general anesthesia,