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Mimicked cartilage scaffolds of silk fibroin/hyaluronic acid with stem cells for osteoarthritis surgery: Morphological, mechanical, and physical clues

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ABSTRACT

Osteoarthritis is a critical disease that comes from degeneration of cartilage tissue. In severe cases surgery is generally required. Tissue engineering using scaffolds with stem cell transplantation is an attractive approach and a challenge for orthopedic surgery. For sample preparation, silk fibroin (SF)/hyaluronic acid (HA) scaffolds in different ratios of SF/HA (*w*/w) (i.e., 100:0, 90:10, 80:20, and 70:30) were formed by freeze-drying. The morphological, mechanical, and physical clues were considered in this research. The morphological structure of the scaffolds was observed by scanning electron microscope. The mechanical and physical properties of the scaffolds were analyzed by compressive and swelling ratio testing, respectively. For the cell experiments, scaffolds were seeded and cultured with human umbilical cord-derived mesenchymal stem cells (HUMSCs). The cultured scaffolds were tested for cell viability, histochemistry, immunohistochemistry, and gene expression. The SF with HA scaffolds showed regular porous structures. Those scaffolds had a soft and elastic characteristic with a high swelling ratio and water uptake. The SF/HA scaffolds showed a spheroid structure of the cells in the porous structure particularly in the SF80 and SF70 scaffolds. Cells could express Col2a, Agg, and Sox9 which are markers for chondrogenesis. It could be deduced that SF/HA scaffolds showed significant clues for suitability in cartilage tissue engineering and in surgery for osteoarthritis.

1. Introduction

Currently, there are many patients who suffer from osteoarthritis. Osteoarthritis is a disease that is the result of cartilage degeneration [1]. The cartilage degeneration leads to a defect of the chondral area. A chondral defect propagates into a large area of the cartilage. In a severe case of osteoarthritis, the patients have pain in their daily lives. In mild cases, the patients usually need to take medication [2,3]. On the other hand, in severe cases, the patients must have surgery by biomaterials replacement [4]. To create new biomaterials using an effective technique and novel technology for surgery is a challenging task for researchers and orthopedic surgeons. Therefore, to create an effective surgical approach for osteoarthritis was chosen for this research.

Tissue engineering is an attractive method that uses the principle of science, engineering, and biomedicine to create novel methods for surgery [5]. Especially, to fabricate new biomaterials that have high effectiveness is interesting for cartilage tissue engineering in osteoarthritis [6]. For a small damaged area of the chondral area, active injectable hydrogels with or without cells have been used to fill the damaged area [7]. Those hydrogels played an important role of inducing cartilage tissue regeneration [8]. On the other hand, for a large area, scaffolds with and without cells were used for cartilage tissue engineering in osteoarthritis [9]. Those scaffolds were put in the chondral defect areas for stability and to induce tissue regeneration. An interesting and attractive method is to use scaffolds with cells for cartilage tissue engineering, and especially to add stem cells into the scaffold and culture them until they differentiate into chondrocytes that would lead to cartilage regeneration. Differentiating stem cells in scaffolds as native cartilage tissue is an important step before transplanting the scaffolds into a chondral defect site of osteoarthritis. Therefore, to engineer scaffolds with stem cells and culture them as native tissue is a challenge to create a performance approach for osteoarthritis surgery.

The mimic approach is attractive for tissue scaffold fabrication that can create the function and structure as native tissue. The mimic approach has been used often to create bio-functionalities for scaffolds [10]. Bio-functional scaffolds can act as potential materials to enhance tissue regeneration [11]. Furthermore, the mimic method was used to construct scaffolds as an extracellular matrix that acted as a native scaffold [12]. The constructed scaffolds based on the mimic approach could

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induce tissue regeneration [13,14]. Due to the attractiveness of the mimic approach, we selected this method to create performance scaffolds with stem cells for cartilage tissue engineering.

Hyaluronic acid (HA) is a natural polymer in the human body particularly in bone joints. HA has antiabrasive and compressive-resistant properties in the bone joint areas. Furthermore, HA has the biofunction as an insoluble signal for tissue regeneration [15]. Due to the unique functionalities of HA, it has been used as a material for tissue engineering. For cartilage tissue engineering, HA was fabricated into an injectable hydrogel with or without encapsulated cells [16–18]. The injectable hydrogel was used to fill a defect or degenerated area of cartilage tissue. The important role of that hydrogel is to resist compressive forces and induce cartilage tissue regeneration. In this research, we selected HA to maintain the bio-functionality of the scaffold. However, because of its instability and biodegradation, HA was blended with other polymers to induce stability [19].

SF is a biomaterial used for various tissue engineering applications because it has good mechanical stability that can maintain the structure of tissue during regeneration [20]. For cartilage tissue engineering, SF was fabricated into scaffolds with and without modification [21,22]. Due to the interesting properties and mechanical stability of SF, we choose it to be the material for scaffolds combined with HA to improve the potential and performance for cartilage tissue engineering.

Keeping in mind the critical challenge of cartilage degeneration, the advantages of HA and SF, and the attractiveness of the mimic approach, the performance scaffold was proposed for cartilage tissue engineering. We fabricated blended SF/HA into 3D porous scaffolds based on the mimic approach. An interesting report presented the salt leaching preparation with genipin crosslinking to combine SF with HA for cartilage scaffold fabrication. The report mainly demonstrated the molecular organization related to the physical performance of the SF scaffold [23]. The SF scaffolds were often incorporated with mesenchymal stem cells (MSCs) for cartilage tissue engineering [24]. This present research focused on the mimicked morphological, mechanical, and physical characteristics of an SF/HA scaffold incorporated with stem cells. The scaffolds incorporated with stem cells were considered to have the potential to induce chondrogenic differentiation. Therefore, as a novel approach in this research we created mimicked structural porous scaffolds that acted as the morphological, mechanical, and physical clues for stem cells to induce cartilage tissue engineering. Eventually, those clues with stem cells were engineered as native cartilage tissue for osteoarthritis surgery.

2. Materials and method

2.1. Preparation of regenerated SF

Silk cocoons from *Bombyx mori* were provided by Queen Sirikit Sericulture Center, Narathiwat, Thailand. Regenerated SF solution was prepared as previously described [1]. Briefly, in the degumming process, raw silk cocoons were boiled twice in 0.25% (*w*/*v*) Na_2CO_3 (Merck or Sigma, 1 g degummed silk/100 mL) for 30 min in order to remove the sericin, waxes, and other impurities and then washed several times with deionized water. The air-dried degummed SF fibers were dissolved in 9.3 M LiBr (Merck 2 g/10 mL) for 4 h at 60 °C. The solution was centrifuged at 4000 rpm for 10 min to remove the insoluble residue and then the solution was dialyzed against deionized distilled water using a dialysis membrane (MW3500, SpectraPor®, USA) for 72 h. The concentration of the obtained SF was determined by Biuret assay and then adjusted to 6% *w*/*v*.

2.2. Preparation of blended SF/HA scaffolds

HA (Sigma-Aldrich) was weighed according to the weight required for fabrication with the silk fibroin in SF/HA ratios (*w*/w) of 100:0 (SF100), 90:10 (SF90), 80:20 (SF80) and 70:30 (SF70) and then dissolved in the purified SF solutions. The blends were incubated under slight agitation with a magnetic stirrer for 1 h at room temperature in order to complete the dissolution of HA and to allow possible interactions between the two components. The total solid weights of SF and HA in the mixtures were controlled at 3% *w*/*v*. After complete mixing, the SF/HA blends were then reacted with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS) (5%*w*/w) for 15 min under mild agitation. Then the mixtures were left for 2 h at room temperature to allow the SF-HA to crosslink. Finally, 2 mL of crosslinked solution were added in each well of a 24-well polystyrene tissue culture plate (TCPS) and frozen at −20 °C overnight prior to lyophilization at −80 °C for 24 h. Additionally, the freeze-dried scaffolds were treated with 80% methanol to stabilize the scaffolds in aqueous solution [25] and also to sterilize the scaffolds.

2.3. Pore size measurement

The pore sizes of the scaffolds in each group were analyzed from SEM images of freeze-dried scaffolds by ImageJ software (v. 1.48). The pore sizes of the scaffolds were determined by randomized sampling ($n = 20$) to calculate the average pore size.

2.4. Morphological structure observation of SF/HA scaffolds

The scaffold morphologies and pore sizes were observed by scanning electron microscopy (SEM-JSM5800LV, JEOL, USA) at an operating voltage of 20 kV for scaffold imaging. In order to observe their structure, the scaffolds were broken vertically in liquid nitrogen and sputter coated with gold prior to investigation by SEM.

2.5. Mechanical properties testing of SF/HA scaffolds

The cylinder shape scaffolds ($n = 6$) were also subjected to unconfined compression tests by using the Universal Testing Machine (Lloyd instruments, LRX-Plus, AMETEK Lloyd Instrument Ltd., Hampshire, UK) equipped with a 10 N capacity load cell. Tests were conducted at room temperature in PBS (wet state) at a constant compression rate of 2 mm min^{-1}. The compressive stress and strain were graphed by NEXYGEN software to measure the sample dimensions of the cross-sectional area and sample height (measured automatically at 0.01 N preload). The compressive modulus and standard derivation were analyzed after testing. The compressive moduli were calculated from the slope between 5% and 10% strain of the compressive stress-strain curve. The values were reported as mean ± standard derivation ($n = 6$).

2.6. Swelling ratio and water uptake testing of SF/HA scaffolds

The swelling ratios of the SF/HA scaffolds were also investigated as previously described [26]. In brief, the dry weight of the scaffolds (W_0) was measured before submersion in PBS for 24 h at room temperature. After removal of excess PBS, the wet weights of the scaffolds (Wt) were measured. The swelling ratios, expressed as $S = (Wt\text{-}Wo)/Wo$, were then calculated where Wo is the initial weight of dried scaffold at time $t = 0$ and Wt is the weight of the hydrated scaffolds at 24 h. The water uptake was expressed as $Wu = [(Wt\text{-}Wo)/Wo] \times 100$. The values were reported as mean ± standard deviation ($n = 6$).

2.7. Cell viability and fluorescence micrograph

In this research, human umbilical cord-derived mesenchymal stem cells (HUMSCs) were used for the cell experiments. Human umbilical cords from both sexes were collected from full-term births after either cesarean section or normal vaginal delivery with informed consent which was approved by the Prince of Songkla University Ethics Committee for Human Research at Songklanagarind Hospital, Hat Yai, Songkhla,