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Silk fibroin/chondroitin sulfate/hyaluronic acid ternary scaffolds for dermal tissue reconstruction

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ABSTRACT

The fabrication of new dermal substitutes providing mechanical support and cellular cues is urgently needed in dermal reconstruction. Silk fibroin (SF)/chondroitin sulfate (CS)/hyaluronic acid (HA) ternary scaffolds (95–248 μm in pore diameter, 88–93% in porosity) were prepared by freeze-drying. By the incorporation of CS and HA with the SF solution, the chemical potential and quantity of free water around ice crystals could be controlled to form smaller pores in the SF/CS/HA ternary scaffold main pores and improve scaffold equilibrium swelling. This feature offers benefits for cell adhesion, survival and proliferation. In vivo SF, SF/HA and SF/CS/HA (80/5/15) scaffolds as dermal equivalents were implanted onto dorsal full-thickness wounds of Sprague–Dawley rats to evaluate wound healing. Compared to SF and SF/HA scaffolds, the SF/CS/HA (80/5/15) scaffolds promoted dermis regeneration, related to improved angiogenesis and collagen deposition. Further, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) expression in the SF/CS/HA (80/5/15) groups were investigated by immunohistochemistry to assess the mechanisms involved in the stimulation of secretion of VEGF, PDGF and bFGF and accumulation of these growth factors related to accelerated wound process. These new three-dimensional ternary scaffolds offer potential for dermal tissue regeneration.

1. Introduction

Efficacious dermal substitutes should provide a template with appropriate pore structure and mechanical support to guide cells, extracellular matrix (ECM) formation and angiogenesis during granulation tissue formation for the treatment of severe skin defects [1,2]. In addition, a microenvironment for stimulating growth factor production and enrichment is an additional design need for dermal substitutes [3].

Many studies have demonstrated that appropriate pore structure and water retention for adhesion, growth and infiltration of cells and new tissue formation were crucial for the successful treatment of skin injury. Interconnected pores can enhance matrix permeability for diffusion of nutrients and waste products, as well as cell cues within the scaffolds [4]. An open-weave scaffold with large pore sizes and high porosity provided for fibroblast infiltration into scaffolds was prepared by electrospinning [5]. Poly(D,L-lactide) scaffolds have inherently small pore sizes that do not allow cell infiltration and ingrowth [6,7] that would be essential for the replacement and repair of dermis [8]. The moist environments effected upon wound healing showed that epithelialization increased 50% in moist environments compared to dry environments [9]. Good water retention in scaffolds to keep the injury zone moist and maintain scaffold shape and fit were critical for the adhesion, differentiation and proliferation of cells [10–12]. Growth factors that accelerate the metabolism of cells and support the reconstruction of the ECM play essential roles in regulating cell fate and tissue formation [13]. For example, bone morphogenetic proteins stimulate bone cell differentiation [14], while fibroblast growth factors and vascular endothelial growth factors stimulate blood vessel differentiation (angiogenesis) [15].

Silk fibroin (SF) is a naturally occurring protein polymer that has been used for centuries in the production of clinical sutures [16]. SF materials support the attachment, proliferation and differentiation of primary cells and cell lines [17–19]. The impressive cytocompatibility and malleability of SF materials make silk a popular starting material for tissue engineering scaffolds used in the repair of many tissues, including skin [20–23]. However, single-component SF scaffolds may not be sufficient for dermal tissue regeneration as there is a shortage of cell specific-binding sites and limited options for growth factor-adsorbing capacity. Hyaluronic acid (HA) is a non-sulfated glycosaminoglycan containing numerous hydrophilic groups that can enhance the hydrophilicity of scaffolds [24], and

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has been added to SF solution to prepare SF-based porous composite scaffolds whose interconnected pores and hydrophilicity were improved compared to SF alone [11,24]. HA, which interacts with cells through receptors on the plasma membrane, such as CD44, contributes to capillary formation [25]. HA or HA-based scaffolds have been studied due to the excellent biocompatibility and water retention [26] and suggested applications for burns and chronic wounds [27–29]. In spite of these interesting features, HA scaffolds present some limitations related to their inadequate mechanical properties that could further affect on their degradation in vivo [11].

Chondroitin sulfate (CS) is glycoprotein with a protein core and polysaccharide branches composed of repeating disaccharide units containing carboxylic and sulfate ester groups [30]. The high negative polarity resulting from the $-SO_4^{2-}$ and $-COO^-$ groups allows CS to be used as a polyanion, onto which positively charged growth factors can be adsorbed and enriched to induce cell adhesion, differentiation and migration [31–34]. Inflammatory reactions at injury sites were reduced by CS due to the accelerated metabolism of cells and the ability to sustain a normal microenvironment for cell growth [35].

We hypothesized that artificial dermal tissue scaffolds with appropriate pore structure, water-binding capacity, degradation rate and growth factor-adsorbing capacity would induce dermal tissue reconstruction in engineered skin tissue in vivo. We present a new bioactive three-dimensional (3-D) tri-copolymer scaffold for dermal tissue reconstruction based on ternary blends of the three biomacromolecules, SF, CS and HA. SF acts as the template of tissue formation, providing mechanical support and controlling the degradation rate. HA enhances water retention and provides a bioactive component for binding vascular endothelial cells to induce mesenchymal stem cells to promote vascularization. CS plays a role as an assembler to adsorb and enrich growth factors. These scaffolds were processed to form porous microstructures that filled the defect domain, improved materials transport and regulated 3-D tissue formation. Finally, groups of scaffolds were grafted onto the dorsum full-thickness wounds of Sprague–Dawley (SD) rats to evaluate the effect of SF/CS/HA (80/5/15) 3-D scaffolds on promoting angiogenesis and dermal tissue reconstruction, as well as to gain insight into the mechanisms involved.

2. Materials and methods

2.1. Materials

Bombyx mori raw silk fibers were purchased from Zhejiang the Second Silk Co. Ltd (Huzhou, China). Chondroitin sulfate, hyaluronic acid sodium ($1.5 \sim 1.8 \times 10^3$ kDa), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 2-morpholinoethanesulfonic acid (MES) and methyl thiazolyl tetrazolium (MTT) dimethyl sulfoxide (DMSO) were all purchased from Sigma Chemical Company (Shanghai, China). L929 cells were provided by the School of Medicine of Soochow University. Dulbecco's modified eagle medium (DMEM, Gibco), fetal bovine serum (FBS, Gibco) and Cell Tracker™ CM-DiI (Molecular Probes, Invitrogen) were purchased from Shanghai Pufei Bio-Technology Co. Ltd, China. All other chemicals were analytical grade and purchased from Shanghai Sinopharm Chemical Reagent Co. Ltd, China, unless otherwise indicated.

2.2. Preparation of SF/CS/HA ternary porous scaffolds

Silk fibroin solution was prepared following the procedure described previously [36]. Briefly, raw silk fibers were degummed three times with 0.05 wt.% (w/w) Na_2CO_3 solution at 100 °C for 30 min, rinsed thoroughly, and dried in an oven. The extracted SF was dissolved in a ternary solvent of $CaCl_2$:CH_3CH_2OH:H_2O (1:2:8 M ratio) at 70 ± 2 °C for 1 h. A 3.2 wt.% SF solution was obtained after dialysis for 4 days followed by filtration. The 1.5 wt.% HA aqueous solution was obtained by dissolving HA powder in deionized water for 24 h. The SF solution was then diluted to 3 wt.% with deionized water. The 3 wt.% SF solution was mixed together with 1.5 wt.% HA solution and then CS powder was added. The final weight ratios of SF/CS/HA in mixed solution were 100/0/0, 80/20/0, 80/15/5, 80/10/10, 80/5/15, 80/0/20, 0/0/100 (w/w/w), respectively. The concentration of final mixed SF/CS/HA solution was diluted to 1.5 wt.%. As reported previously [19,37], the EDC, NHS and MES were added into the solution to account for 20%, 10% and 20% weight ratio against the total weight of SF, CS and HA in solution, respectively. Here, the NHS plays an activation of –OH group; the MES creates a faintly acid condition to be beneficial for the cross-link reaction. The mixed solution was poured into stainless steel dish, frozen at −40 °C for 8 h, followed by lyophillization for 48 h.

2.3. Pore characteristics

The cross-sectional morphology of SF/CS/HA porous scaffolds was observed with a Hitachi S-4800 scanning electron microscope (SEM, Tokyo, Japan). The border of each pore in the top layer was defined according to a gradient method. The images of the top layer of the cross-sections were obtained. The total area S (mm^2) in the visual field and each pore area ($S_1, S_2, \ldots, S_i, \ldots, S_n$) were calculated according to the limits of each pore in bitmaps and the number of image points in the whole image. The pore diameter d_i (μm) of each pore, average pore diameter $\overline{d}$ (μm) and porosity P (%) are given by Eqs. (1)–(3) [38]:

$$d_i = \sqrt{\frac{4s_i}{\pi}} \tag{1}$$

$$\overline{d} = \frac{\sum_{i=1}^{n} d_i}{n} \tag{2}$$

$$P = \frac{\sum_{i=1}^{n} s_i}{S} \times 100 \tag{3}$$

2.4. Swelling properties

The swelling properties were measured according to a previous method [26]. SF/CS/HA ternary scaffolds were cut to 20 × 20 mm (length × width) and immersed in distilled water at room temperature for 24 h. After excess water was removed, the wet weight of the scaffold W_s (g) was determined. Samples were then dried in an oven at 65 °C under vacuum overnight and the dry weight of scaffolds W_d (g) was determined. The swelling ratio Q (g/g) of the ternary scaffolds was calculated by Eq. (4):

$$Q = \frac{W_s - W_d}{W_d} \tag{4}$$

2.5. Cell viability assay

Viability of cells was investigated by MTT assay and laser scanning confocal microscopy (LSCM, TCS-SP2, Leica Company, Germany). First, the round porous SF/CS/HA scaffolds with the diameter of 15 mm were placed in 24-well plates (Corning Inc., New York, USA), sterilized by γ-ray irradiation followed by rinsing with phosphate-buffered saline (PBS) prior to cell seeding. L929 cells at a density of 1×10^5 cells ml^{-1} were seeded onto the scaffolds in 24-well plates in DMEM with 10% FBS. The cell-seeded