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Three-dimensional electrospun silk-fibroin nanofiber for skin tissue engineering

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ABSTRACT

Tissue-engineered skin substitutes may offer an effective therapeutic option for the treatment of patients with skin damages. In this study, a novel three-dimensional (3D) scaffold composed of electrospun silk fibroin (SF) nanofiber was fabricated using electrospinning with the addition of NaCl crystals. It has well known that the electrospun SF nanofibers were excellent scaffold for tissue. However, it is generally difficult for cells to infiltrate the electrospun silk fibroin due to its small pore size. To resolve this problem, we dropped the NaCl crystals above the rotating collector, which become incorporated into the nanofibers. Three methods (freeze-drying, salt-leaching, and electrospinning with NaCl) for fabrication of SF scaffolds were compared to the difference of their characteristics using scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), mechanical strength, porosity, swelling abilities, and cell proliferation. Additionally, using air-liquid culture system, keratinocytes were co-cultured with fibroblasts in each type of SF scaffolds to construct an artificial bilayer skin *in vitro*. In our experimental results, histologic findings in only electrospun SF scaffolds showed more proliferation of fibroblasts in deep layer and more differentiation of keratinocytes in superficial layer. The present study suggests that 3D electrospun SF scaffolds might be a suitable for skin tissue engineering.

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1. Introduction

Skin is composed of two primary layers: the epidermis, which serves as a barrier to infection and the dermis, which serves as a location for the appendages of skin. The epidermis is thin and an outer-layer that contains keratinocytes. The dermis is relatively thick and an inner layer that contains fibroblasts and extracellular matrix (ECM). Thus, this layer provides strength, flexibility, and networks systems for body homeostasis [1].

Sometimes, skin defects caused by congenital defect, burn, trauma, or disease and affect individual physical and psychological well-being status. In the United States, more than 2 million patients need medical care following burns. Among them, about 2% patients will suffer severe burns and necessitate some skin graft [2]. However, available skin donor sites may be very limited in extensive burn patients and also simple autografting are often not enough. Additionally, various commercially available products can usually provide a temporary effect for coverage of skin defects. Therefore, treatment of skin defects is still one of the challenging problems in plastic, reconstructive, and burn fields.

Recently, biotechnological progress suggests that tissue engineering is an interesting approach to counteract organ deficit and collagen is known to be one of the most effective biomaterials in various tissue engineering. Until now, some collagen-based dermal substitutes, such as Dermagraft (Advanced Tissue Sciences Inc., USA) [3] and Apligraf (Organogenesis, USA) [4] have been used in these fields for the skin substitutes. However, because of the antigenicity or the wound contraction of donor sites, the dermal substitutes usually give us an unsatisfactory outcome. Moreover, collagen has the crucial limitation for skin tissue engineering, including fast degradation rate, low mechanical strength, and expensive price. Therefore, in the past decades, various synthetic or natural polymers have been investigated for skin tissue engineering, including collagen sponge with silicone film [5], chitosan film and sponge [6], chitosan/gelatin [7], gelatin/chondroitin-6-sulfate/hyal-uronic acid

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[8], and Polycaprolactone (PCL)/Poly(lactic-co-glycolic acid) (PLGA) with chitosan/gelatin hydrogel [9] for skin tissue engineering applications.

Silk fibroin (SF), a natural protein produced by *Bombyx mori*, is highly biocompatible, minimally immunogenic, non-toxic, non-carcinogenic, and biodegradable. Recently, the SF had an intensive attention as a material for three-dimensional (3D) porous scaffold because of its excellent intrinsic properties, biocompatibility, biodegradability and mechanical strength [10–15]. Many studies have been reported that the 3D SF scaffolds provides excellent support for cell proliferation and can also use for various tissue engineering, including bone, cartilage, and blood vessel [16–25]. Until now, there are several methods to fabricate the porous 3D SF scaffolds including freeze-drying, salt-leaching, and electrospinning [26–36].

Among those, electrospinning technique has distinct advantages as a skin scaffold than the conventional 3D scaffolds like its similarity with extracellular matrix (ECM). Because the structural and physical properties of electrospun nanofibers resemble those of an ECM, electrospun nanofibers of synthetic or regenerated natural polymers promote cell proliferation, enhanced nutrient, and waste transfer. Thus, 3D electrospun nanofibers scaffolds have been widely used as scaffolds for various tissue engineering applications [37]. One paper showed that nonwoven SF nanofibrous nets were demonstrated to support the adhesion, proliferation, and cell–cell interactions of variable human cell lines including osteoblasts, fibroblasts, keratinocytes, and endothelial cells [19]. Additionally, another studies described that nonwoven SF nanofibrous nets could support cell adhesion, spreading and proliferation in different cell types *in vitro* [38,39]. In our previous study, electrospun SF showed a wound healing effect in full thickness skin defects in rats [40]. Above these studies suggest that the 3D electrospun SF nanofiber scaffold has an enough potential as a good candidate for the skin substitute.

Therefore, the aim of the present study was to develop the skin substitute by using the 3D electrospun SF nanofiber scaffold. This study was co-culture keratinocytes with fibroblasts in the 3D electrospun SF nanofiber scaffold to produce a bilayer skin substitute *in vitro*. In addition, we compare to the difference of characteristics and cell culture between methods for SF scaffold: freeze-drying, salt-leaching, and electrospinning.

2. Materials and methods

2.1. Preparation of B. mori silk fibroin solution

Silkworm cocoons were obtained from the Rural Development Administration (Suwon, Korea). To remove the sericines, *Bombyx mori* cocoons were degummed by boiling for 30 min in an aqueous Na_2CO_3 solution and then washed with distilled water several times. Subsequently, degummed silk was dissolved in $CaCl_2$: H_2O: ethanol in a molar ratio of 1: 8: 2. And then, this solution was filtered through a miracloth (Calbiochem, San Diego, CA) and dialyzed against distilled water for 4 days. Silk fibroin solutions were stored at 4 °C before use to avoid premature precipitation. The final concentration of the aqueous silk fibroin solution was 10.0 w/v%. The SF solutions were stored at 4 °C before use to avoid premature precipitation.

2.2. Preparation of silk scaffolds using freeze-drying or salt-leaching method

For fabrication of silk scaffolds, in freeze-drying method, the 10.0 w/v% SF solution was adjusted to 2.0 w/v% and then put in a deep-freezer for 6 h. Next line, this was lyophilized for 12 h and the samples were subjected to ethanol treatment. After this, the samples were stored in dry place and were made of small discs (2 mm in diameter and 6 mm in thickness) using a biopsy punch (Fig. 1).

Meanwhile, a schematic diagram of silk scaffolds using salt-leaching method was shown in Fig. 2. In brief, firstly, the 50 g of salt particles (particle size: 250–300 μm) were spread out on petri dish regularly. And then, 10.0 w/v% silk fibroin solutions were poured into petri dish. Immediately after pouring SF solutions, the petri dish was filled with adding 100 g of salt particles. After that, the petri dish was immersed in distilled water at room temperature for 72 h to extract the salt particles. Then, silk scaffolds were punched for design of small discs (2 mm in diameter and 6 mm in thickness) and were placed into 2.0 w/v% SF solution. Finally, these were lyophilized in a freeze-dryer (Genesis 25-LE, Virtis) for 12 h.

2.3. Salt leaching electrospining(SLE)

SF solution was blended with polyethylene oxide (PEO) to obtain desirable viscosity and spinnability. Silk/PEO (mixture ratio: 4:1) aqueous solution was prepared by adding PEO (200,000 MW, Sigma Aldrich, St. Louis, MO, USA) directly into the SF aqueous solution of 5.8 wt.%. Electrospinning was performed by placing the silk/PEO in a 10-mL plastic syringe with a 22 needle gauge (0.7 mm OD × 0.4 mm ID) at a constant flow rate of 1.2 ml/h, which was maintained using a syringe pump that was mounted on an adjustable stand to keep the solution at the tip of the needle without dripping, as described elsewhere [41,42]. The positive electrode was used to apply a voltage of +20 kV to the needle tip through an alligator clip, while the negative electrode was set to an applied voltage of −2 kV to the collector. Four syringes were mounted in parallel plate geometry, yielding nanofibers that were used for the electrospinning. The needle tip-to-collector distance was 15 cm. Such processing parameters were accurately adjusted so that stable jets could be obtained. To fabricate the 3D electrospun SF scaffolds(ESF), NaCl crystals (with a diameter of 250–300 μm) were released from a rotating cylinder above the drum collector (Fig. 3). The release rate of NaCl was approximately 35 g/h. The SF nanofiber matrices were immersed in 95% ethanol for 30 min and then placed in deionized water to allow the NaCl and PEO to leach out for one day; the water was changed three times. The ESF using SLE was made by lyophilized in a freeze-dryer (Genesis 25-LE, Virtis) for 48 h.

2.4. Characterization

2.4.1. Scanning electron microscopy (SEM)

The cross-sectional morphology of 3D-silk scaffolds was investigated with an environmental scanning electron microscope (E-SEM, S-3500N, Hitachi, Tokyo, Japan) in low vacuum mode at the Korean Basic Science Institute, Chuncheon. Before viewing, the samples were pasted on a carbon tape and sputter-coated using a thin layer of gold palladium for 120 s for two consecutive cycles at 45 mA with the Ion Sputter 1010 (Hitachi, Tokyo, Japan). After sample coating, the micrographs from each samples were taken at an accelerating voltage of 2 KV and with magnifications of 15 K. The morphology of pore was evaluated in the cross section of the scaffolds. Three samples were tested for each type of scaffolds.

2.4.2. Fourier transform infra-red spectroscopy (FTIR)

To indentify the infrared spectra of SF structures in scaffolds, Fourier transform infrared spectroscopy (FTIR) analysis was performed by using BIO-RAD (Cambridge, MA, USA). The samples were directly loaded on ATR window, and spectra were collected using Excalibur Series by averaging 32 scans with the resolution of 4 cm^{-1}.

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