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Functionalized silk fibroin dressing with topical bioactive insulin release for accelerated chronic wound healing

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ABSTRACT

The healing of chronic wounds remains a key challenge in regenerative medicine. To promote wound healing, a bioactive wound dressing is required. In this study, a functionalized silk fibroin dressing with topical bioactive insulin release was prepared for the treatment of chronic wounds. For this purpose, insulin-encapsulated silk fibroin (SF) microparticles were prepared by coaxial electrospraying of aqueous SF solution under mild processing conditions. Insulin was successfully encapsulated in the inner layer of SF microparticles, providing a sustained insulin release for up to 28 days. It was found that the insulin released from the microparticles could maintain original molecular conformation. Moreover, the cell migration assay based on human keratinocyte and endothelial cells confirmed that the insulin released from SF microparticles retained its native bioactivity. Furthermore, the insulin-encapsulated microparticles were loaded into a SF sponge, functioned as a bioactive wound dressing, and the *in vivo* therapeutic effect of the sponge dressing was evaluated on dorsal full thickness wounds of diabetic Sprague-Dawley rats. The results showed that an insulin-functionalized SF dressing accelerated wound closure, collagen deposition and vascularization, thus, significantly promoting wound healing. The insulin-functionalized SF dressing provides new treatment options for chronic wounds.

1. Introduction

Chronic cutaneous wounds are characterized by the absence of healing after 6 weeks, which is commonly seen as a consequence of diabetes mellitus and vascular compromise [1,2]. The classic treatment is the debridement of the wound followed by its compression with sterile gauze. When this method is not effective enough in some chronic wounds, the dressings based on adequate biomaterials are frequently used to promote healing [1]. However, treatment currently focuses on dressings that prevent microbial infiltration and keep a balanced moisture and gas exchange environment. To heal some chronic wounds, a bioactive dressing is frequently required. For example, in the cutaneous wound in diabetic foot ulcers, reepithelialization and angiogenesis are powerless. Therefore, functionalized wound dressings with sustained bioactive drug release are needed to offer a stimulatory function for facilitating tissue repair, thus, promoting wound healing and minimizing the recovery period [3,4].

Recently, increasing evidences demonstrated that insulin contributes to wound healing [5–7]. It was reported that insulin accelerated reepithelialization of provisional tissue by stimulating the migration and proliferation of keratinocyte [4,8,9]. In addition, insulin can stimulate the migration and tube formation of endothelial cells which helps to improve angiogenesis during wound healing [9,10]. However, the major problem of topical administration of peptides is their short half-life and loss of bioactivity in the peptidase-rich wound environment [11]. An alternative strategy to overcome this problem is the use of biocompatible wound dressings for sustained delivery of insulin.

Drug delivery using biodegradable microspheres is a promising approach for sensitive biologicals, and insulin has been encapsulated into polymer microparticles to establish a sustained delivery system [4,6,12,13]. However, most methods for the preparation of insulin delivery microparticles, such as emulsification and the solvent extraction method [9,12], need processing in organic solvents, at extreme pH values, or mechanical stress, potentially challenging the bioactivity of insulin [14,15]. Unlike other small-molecule drugs, insulin possessing a complex molecular conformation for its biological activity is more susceptible to salts, organic solvents and high temperature, which may cause a loss of bioactivity [16]. In this regard, the retention of bioactivity of insulin released from dressing remains the key challenge.

As a natural protein, silk fibroin (SF) has been explored for various tissue engineering applications due to excellent biocompatibility and tailorable degradability [17,18]. SF materials showed excellent bioresponses *in vivo* with low immunogenicity for numerous clinical applications [19]. SF biomaterials have been applied as wound healing dressings in diverse structural forms such as film, sponge and

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electrospun fibers, which supported cell growth of fibroblasts, keratinocytes and endothelial cells, and showed a positive effect for wound healing [18–24]. In addition, silk is generated in an aqueous state and is thus readily miscible with other protein solutions. Its aqueous solubility and processability under very mild conditions makes it an attractive material for the loading of sensitive biologics to avoid loss of bioactivity of the drugs to be delivered [25,26]. SF-based biomaterials are being considered to address a wide range of stabilization challenges, from labile enzymes to antibodies [27]. Glucose oxidase, lipase, and horseradish peroxidase were entrapped in SF films over 10 months and significant activity was retained, even when stored at 37 °C [27]. The SF microparticles loaded with insulin-like growth factor 1 demonstrated controlled and sustained release over 7 weeks in bioactive form [26].

Therefore, we hypothesized that insulin-encapsulated SF microparticles are able to provide a sustained release of bioactive insulin, and the microparticle-loaded SF dressing can promote the migration of skin repair cells and the reconstruction of a microvascular network for healing chronic wounds. In the present study, we prepared insulin-encapsulated SF microparticles by coaxial electrospraying and freeze-drying. This method proposed to prepare SF microparticles under very mild conditions from aqueous SF solutions, which facilitates maintaining the bioactivity of insulin. Then, the insulin-encapsulated microparticles that offered a topical and sustained release system would be incorporated into SF sponges *via* multilayer loading, functioning as a bioactive wound dressing for chronic wounds. The molecular conformation of insulin released from microparticles was detected by FTIR and CD, and the bioactivity of released insulin was assessed by the scratch assay of the immortal human keratinocyte (HaCaT) and the human endothelial cell (EA. hy926). Furthermore, the *in vivo* therapeutic effect of SF dressings, including healing rate and vascularization, was evaluated on dorsal full thickness wounds of diabetic Sprague-Dawley (SD) rats.

2. Materials and methods

2.1. Preparation of SF solution

Regenerated SF solution was prepared following the procedure described previously [18]. Briefly, *Bombyx mori* silk fibers (Huzhou, China) were degummed three times in 0.05% Na_2CO_3 solution at 98–100 °C for 30 min and dried at 60 °C after thoroughly rinsing. The extracted silk fibroin was dissolved in a ternary solvent of $CaCl_2$:CH_3CH_2OH:H_2O (1:2:8 M ratio) at 72 ± 2 °C for 1 h. SF solution was obtained after dialysis with cellulose membranes (MWCO 9–12 kDa) in deionized water for 4 days.

2.2. Preparation of SF microparticles

100 mg insulin (27.5 IU/mg; from porcine, WangBang Bochemical Pharmaceutical Co., Ltd., Xuzhou, China) was dissolved in 10 mL of 0.01 M HCl, then the pH of the solution was adjusted to 7.0 ± 0.1 using 0.1 M NaOH. SF solution was diluted to 2.0 wt% and mixed with glycerol at 30 wt% of SF weight. The resulting insulin solution (1.0 wt%) and SF solution (2.0 wt%) were used as core and shell for coaxial electrospraying, respectively.

Fig. 1 (A) shows the preparation process for insulin-encapsulated SF microparticles. The coaxial nozzle has an inner capillary of 0.6 mm (inner diameter) and an outer capillary of 1.2 mm (inner diameter). Two syringe pumps (KDS100, KD Scientific, USA) deliver core and shell layer solutions at the rates of 0.1 mL/h and 0.3 mL/h, respectively. A high-voltage power supply supplied a 13 kV high voltage between the nozzle and the collection box filled with liquid nitrogen, and the distance is 12 cm. The collected insulin-encapsulated SF microparticles were lyophilized by a Virtis Genesis 25-LE Freeze Dryer for 48 h and then balanced at 25 °C and RH 90% for 24 h to obtain the water-insoluble microparticles. Drug loading ratio was expressed as the percentage of theoretical drug amount relative to entire microparticles. Eq. (1) was used to evaluate insulin loading content.

$$\text{Insulin loading } (\%) = \frac{\text{Weight of the insulin in micropaticles (mg)}}{\text{Weight of the micropaticles (mg)}} \times 100 \quad (1)$$

The loading ratio of insulin in the coaxial microparticles was about 16.7% according to the above equation. As a control, the pure SF and insulin microparticles were prepared, respectively, the solution delivering rate was 0.4 mL/h and the other conditions remained consistent.

2.3. Preparation of microparticle-loaded SF sponge dressing

As shown in Fig. 1 (B), a microparticle-loaded SF sponge dressing was prepared by multilayer loading and freeze-drying method. The SF solution was diluted to 2.0 wt%, and then 2-morpholinoethanesulfonic acid (MES), *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Sigma-Aldrich) were added to the SF solution at 20%, 10% and 20% of the SF weight in solution, respectively. The mixed solution was stirred slowly and reacted in an ice bath for 1 h. 900 μL of EDC-activated SF solution was added into a round metallic box (D = 40 mm, H = 15 mm, V = 10 mL), and frozen at −80 °C for 30 min. Then 10 mg of SF microparticles were evenly laid on a frozen SF solution layer, and frozen at −80 °C for 15 min. On top of it, 700 μL of EDC-activated SF solution was poured to cover the microparticles and frozen at −80 °C for 15 min. Finally, 10 mg of microparticles were added and frozen, and subsequently, 900 μL of EDC-activated SF solution was poured according to the above process. Then the SF mixture composite with two layers of microparticles were frozen at −80 °C for 4 h, and further lyophilized to obtain a microparticle-loaded sponge dressing. The dressings were sterilized with γ-ray irradiation and stored at 4 °C.

2.4. Scanning electron microscopy

The morphology of samples was observed by a scanning electron microscopy (SEM, S-4800, Hitachi, Japan). The size of microparticles was analyzed on the basis of SEM images with the Nano Measurer analysis software (Department of Chemistry, Fudan University, China. Copyright: (C) 2008 Jie Xu). To calculate the diameter of microparticles, we measured the average diameter of a total of 100 microparticles based on using SEM images.

2.5. In vitro insulin release

Insulin was labeled with fluorescein isothiocyanate (FITC) (Sigma-Aldrich) as previously described [28]. 300 μL of FITC solution (10 mg/mL in dimethylsulfoxide) was added to 10 mL of insulin solution (15 mg/mL in bicarbonate buffer, pH = 8.5, 0.1 M) and stirred at room temperature for 60 min. Next, 200 μL of 1 M hydroxyl ammonium chloride solution was added and stirred for 10 min at room temperature. The insulin was then purified using a 10 mm × 300 mm column with Sephadex G-50 equilibrated in 0.1 M sodium bicarbonate buffer (pH = 8.5) to remove any unreacted FITC. FITC-insulin encapsulated SF microparticles were prepared according to the condition found in Section 2.2. The fluorescent images were captured using an inverted fluorescence microscope (Olympus IX71, Japan). Physically absorbed microparticles were used as a control group. 10 mg of pure SF microparticles was directly immersed into 2 mL of FITC-insulin solution to adsorb insulin by permeating and physical absorption, and named as insulin-adsorbed microparticles. The remaining amount of insulin in solution was quantified to calculate the adsorption amount using a fluorescence spectrophotometer (FM4P TCSPC, Horiba Jobin Yvon), and the loading ratio in the insulin-adsorbed microparticles was about 5.5%.