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Novel bilayer wound dressing based on electrospun gelatin/keratin nanofibrous mats for skin wound repair

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ABSTRACT

A bilayer membrane (GKU) with a commercial polyurethane wound dressing as an outer layer and electrospun gelatin/keratin nanofibrous mat as an inner layer was fabricated as a novel wound dressing. Scanning electron micrographs showed that gelatin/keratin nanofibers had a uniform morphology and bead-free structure with average fiber diameter of 160.4 nm. 3-(4,5-Dimethylthiazolyl)-2,5-diphenyltetrazolium bromide assay using L929 fibroblast cells indicated that the residues released from the gelatin/keratin composite nanofibrous mat accelerated cell proliferation. Cell attachment experiments revealed that adhered cells spread better and migrated deeper into the gelatin/keratin nanofibrous mat than that into the gelatin nanofibrous mat. In animal studies, compared with the bilayer membrane without keratin, gauze and commercial wound dressing, Comfeel®, GKU membrane gave much more number of blood vessels and a greater reduction in wound area at 4 days, and better wound repair at 14 days with a thicker epidermis and larger number of newly formed hair follicles. GKU membrane, thus, could be a good candidate for wound dressing applications.

1. Introduction

Skin, the largest organ of the human body, is easily damaged by wounding and burning [1]. The healing of large full-thickness skin defects remains a significant clinical problem. Numerous skin substitutes such as autografts, allografts, and xenografts have been shown to enhance wound repair [2]. However, some problems associated with these naturally derived skin substitutes are the limited number of donor sites available and antigenicity [3]. In recent years, wound dressings based on biopolymer nanofibers with native extracellular matrix-(ECM) like fibrous architectures provide an emerging approach for treatment of full-thickness skin wounds [4,5].

Mammalian cells in vivo are in close contact with the ECM macromolecules comprised of networks of fibers ranging in diameter from 50 to 500 nm [6]. Scaffolds with nanostructure, mimicking native tissue environment, are beneficial for cell adhesion, proliferation, and differentiation [7]. Among the various techniques used to fabricate nanofibrous membranes, the electrospinning technique provides a simple and efficient way to produce fibers ranging from nanometer to sub-micrometer in diameter, depending on the solution and process parameters [8,9]. The solution viscosity, surface tension, flow rate, applied voltage, tip to target distance, surrounding humidity, and temperature play important roles in controlling fiber formation and morphology [10,11]. The electrospun nanofibrous membranes could provide high specific surface areas with adequate porosity and three-dimensional network structures to enhance cell adhesion, migration, and growth [12]. Therefore, electrospun nanofibers are good candidates for a variety of biomedical applications, including drug delivery, wound dressings, and serving as scaffolds for tissue engineering [13–15].

To date, numerous polymers, including synthetics non-degradable and degradable polymers as well as biopolymers, have been electrospun to produce nanofibrous membranes for skin tissue engineering [16–18]. Gelatin is derived by denaturing collagen and has almost identical composition and biological properties as those of collagen. However, gelatin has a lower antigenicity than collagen [19]. It possesses the arginine–glycine–aspartic acid (RGD) sequences of collagen, making it highly effective for cell adhesion [20]. Moreover, it contains large amounts of glycine, proline, and hydroxyproline that potentially accelerate soft tissue healing and stimulate wound healing [21,22]. Our previous study

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demonstrated that the nanofibrous structure of gelatin fabricated by an electrospinning technique could enhance cell adhesion and proliferation [23]. Therefore, gelatin-based nanofibers have a potential for use in tissue engineering and wound dressing. However, the mechanical properties of the pure gelatin nanofibrous membranes are very poor [24,25]. Poly(vinyl alcohol) (PVA) is a non-toxic, water-soluble synthetic polymer with good physical and chemical properties, which has been widely used in biomedical applications, such as wound dressing and drug delivery system. The introduction of PVA in the gelatin/PVA fibrous scaffolds could improve the flexibility [24]. Moreover, Yang et al. demonstrated that the tensile strength and elongation at break of the gelatin/PVA nanofibrous membranes increased as the ratio of PVA increased, because of the good deformability and flexibility of PVA [25]. Moreover, it was found that PVA could significantly improve the spinnability of gelatin [26].

Keratin is a fibrous structural protein found abundantly in skin, hair, wool, nails, claws, horns, hooves, and feathers. The presence of disulfide bond makes keratin good mechanical durability [27]. The addition of keratin to gelatin would provide a better mechanical strength [28]. Additionally, keratin-based materials are suitable for chronic wound dressing due to their interaction with the proteolytic wound environment to accelerate wound healing [29]. Moreover, keratin protein extracted from hair and wool fibers contains cell adhesion sequences, RGD and leucine–aspartic acid–valine (LDV), which are also found in several ECM proteins like fibronectin [30,31]. Due to the presence of such sequences, keratin is able to support cell attachment and proliferation. Yamauchi et al. exhibited that the keratin was more adhesive to fibroblasts and enhanced more cell proliferation than the type I collagen [32]. Compared to alginate/gelatin hydrogels, alginate/keratin hydrogels promoted fibroblast growth and spreading [33]. These advantages make keratin attractive as tissue engineering constructs [34,35]. The major drawback of keratin-based scaffolds and films is their brittleness [36]. Therefore, some researchers blended keratin with other components to form new wound dressings [37–39]. For example, Thilagar et al. prepared keratin-gelatin composite film and used it to treat the full-thickness wounds in dogs [40]. They found that the composite film could enhance early formation of hair follicles, sebaceous gland and required epidermal thickness compared with basic fibroblast growth factor-impregnated gelatin film. Moreover, keratin has been *co*-electrospun with several synthetic polymer, such as poly(hydroxybutylate-*co*-hydroxyvalerate), polylactide, and poly(ε-caprolactone), as tissue engineering scaffolds and wound dressings [41–43]. To the best of our knowledge, however, there has been no report on preparing gelatin/keratin composite nanofibrous membrane by electrospinning, and use it as a wound dressing.

In the present study, keratin extracted from human hair was blend with gelatin and electrospun on top of a commercial polyurethane (PU) wound dressing to produce a bilayer membrane. The PU outer layer acts as a barrier to bacteria and external contaminants. The gelatin/keratin composite nanofibrous mat as an inner layer of wound dressing was designed to mimic the structure of the natural ECM. The morphology and in vitro degradation of the nanofibrous mat were examined. 3-(4,5-Dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine its cellular compatibility. The influence of nanofibrous structures on cellular response was investigated by scanning electron microscope (SEM) and confocal microscope observation. Finally, the effect of the membrane on wound healing in rat was evaluated.

2. Materials and methods

2.1. Extraction of keratin from human hair

Keratin was extracted from human hair according to the Shindai method [44]. Briefly, hair collected from local barber shops in Taichung, Taiwan was washed thoroughly with 70% ethanol and deionized water in sequence, and dried in air. 100 mg of dry hair was mixed with 5 mL of 25 mM Tris–HCl solution (pH 8.5) containing 2.6 M thiourea, 5 M urea and 5% 2-mercaptoethanol at 50 °C for 3 days to extract keratin. The resulting mixture was than filtered and centrifuged at 15,000 ×*g* for 20 min at room temperature. The obtained supernatant was dialyzed against deionized water using cellulose tubing with a 6–8 kDa molecular weight cutoff for 1 day at room temperature. Finally, the dialyzed solution was freeze-dried to yield a keratin powder.

2.2. Electrospinning of gelatin/keratin nanofibers

Gelatin powders (Bloom number 300, Sigma-Aldrich, St. Louis, MO) was dissolved in formic acid (88 wt%) with a concentration of 17 wt%. PVA (Mw = 1400, Showa, Japan) was dissolved at a concentration of 10 wt% in hot deionized water. The PVA aqueous solution was then added to the gelatin solution with a gelatin/PVA ratio of 9/1 (v/v) [23]. After that, keratin powders was dispersed into the mixed solution at a concentration of 2 μg/mL and stirred overnight at room temperature to ensure complete dissolution. The blended polymer solution was loaded into a syringe with a metal needle (G22, diameter = 0.41 mm) and then spun toward a commercial PU wound dressing (HeraDerm®; Amed, New Taipei City, Taiwan) for 2 h using the electrospinning machine (SC-80H, Xian Xing Electric Co., Taiwan) to form a bilayer GKU membrane. The typical parameters for electrospinning experiments were as follows: 20 kV (voltage), 10 cm (tip-to-collector distance) and 0.1 mL/h (feed rate). After electrospinning, the fibers were cross-linked in 50 wt% glutaraldehyde vapor for 45 min. Fibrous mat without keratin was also electrospun on PU membrane (GU) as control. The thickness of nanofibrous mat was about 160 μm.

2.3. Fiber morphology observation

The morphology of the gelatin/keratin composite nanofibers was investigated by SEM (Hitachi S-3000 N, Tamura, Japan) at an accelerating voltage of 20 kV. Prior to imaging by SEM, the sample was sputter-coated with gold. The averaged diameter of the electrospun fibers was determined using the Image J software (National Institute of Health, Bethesda, MD).

2.4. Water uptake capability

The dry gelatin/keratin composite nanofibrous mat with or without PU membrane as well as dry PU membrane only sample were cut into 1.5 cm × 1.5 cm squares and soaked in phosphate buffer solution (PBS, pH 7.4). After soaking for 3, 6, 12, 24 and 48 h at 37 °C, the swollen specimen was removed from PBS, gently blotted with a filter paper to remove excess surface liquid, and immediately weighed (W_{wet}). The specimen was then lyophilized and weighed again (W_{dry}). The water uptake percentage ($\Delta W\%$) at each time point was calculated using the formula: $\Delta W\,(\%) = (W_{wet} - W_{dry})/W_{dry} \times 100\%$.

2.5. In vitro degradation of nanofibrous mat

The gelatin/keratin composite nanofibrous mat (1.5 cm × 1.5 cm) without PU membrane was lyophilized and weighed (W_i). Subsequently, the specimen was incubated in PBS at 37 °C. After incubation for 1, 2, 3 and 4 weeks, the specimen was taken out of PBS, lyophilized and weighed again (W_f). The weight loss percentage ($\Delta W\%$) at each time interval was determined according to the following equation: $\Delta W\,(\%) = (W_i - W_f)/W_i \times 100\%$.

2.6. Cytotoxicity of extracts from nanofibrous mat

Following sterilization with ^{60}Co gamma ray irradiation (15 kGy), the gelatin/keratin composite nanofibrous mat was placed in a sterilized tube that was filled with aseptic deionized water and incubated. After soaking for 1, 2, 3 and 4 weeks at 37 °C, the extracts were collected for