Acta Biomaterialia 39 (2016) 146–155

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Multi-biofunction of antimicrobial peptide-immobilized silk fibroin nanofiber membrane: Implications for wound healing

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ARTICLE INFO

Article history:
Received 30 December 2015
Received in revised form 18 April 2016
Accepted 4 May 2016
Available online 6 May 2016

Keywords:
Antimicrobial peptide
Immobilization
Wound dressing
Silk fibroin
Nanofiber

ABSTRACT

An antimicrobial peptide motif (Cys-KR12) originating from human cathelicidin peptide (LL37) was immobilized onto electrospun SF nanofiber membranes using EDC/NHS and thiol-maleimide click chemistry to confer the various bioactivities of LL37 onto the membrane for wound care purposes. Surface characterizations revealed that the immobilization density of Cys-KR12 on SF nanofibers could be precisely controlled with a high reaction yield. The Cys-KR12-immobilized SF nanofiber membrane exhibited antimicrobial activity against four pathogenic bacterial strains (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa*) without biofilm formation on the membrane surface. It also facilitated the proliferation of keratinocytes and fibroblasts and promoted the differentiation of keratinocytes with enhanced cell-cell attachment. In addition, immobilized Cys-KR12 significantly suppressed the LPS-induced TNF-α expression of monocytes (Raw264.7) cultured on the membrane. These results suggest that a Cys-KR12-immobilized SF nanofiber membrane, which has multiple biological activities, would be a promising candidate as a wound dressing material.

Statement of Significance

This research article reports various bioactivities of an antimicrobial peptide on electrospun silk fibroin nanofiber membrane. Recently, human cathelicidin peptide LL37 has been extensively explored as an alternative antibiotic material. It has not only a great antimicrobial activity but also a wide variety of bioactivities which can facilitate wound healing process. Especially, many studies on immobilization of LL37 or its analogues have shown the immobilization technique could improve performance of wound dressing materials or tissue culture matrices. Nevertheless, so far studies have only focused on the bactericidal effect of immobilized peptide on material surface. On the other hand, we tried to evaluate multi-biofunction of immobilized antimicrobial peptide Cys-KR12, which is the shortest peptide motif as an analogue of LL37. We fabricated silk fibroin nanofiber membrane as a model wound dressing by electrospinning and immobilized the antimicrobial peptide. As a result, we confirmed that the immobilized peptide can play multi-role in wound healing process, such as antimicrobial activity, facilitation of cell proliferation and keratinocyte differentiation, and inhibition of inflammatory cytokine expression. These findings have not been reported and can give an inspiration in wound-care application.

1. Introduction

Wound treatment is one of the most important and challenging healthcare issues. In the United States, 6.5 million patients suffer from chronic wounds and spend 25 billion US dollars annually on their treatment [1]. Without proper treatments, skin wounds are often exposed to bacterial infection, which prolongs inflammation,

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http://dx.doi.org/10.1016/j.actbio.2016.05.008
1742-7061/

disturbs re-epithelialization, inhibits collagen production, and delays wound healing [2]. In addition, once bacteria adhere to a solid surface, they form biofilms, which are sessile communities of bacteria that are embedded with extracellular polymeric substances. These biofilms protect bacteria from the immune system and antibiotics and release endotoxins that cause sepsis, which can lead to death [2,3]. Therefore, preventing bacterial infection and biofilm formation is of utmost importance in wound treatment.

In wound treatment, wound dressing is typically used to facilitate wound healing. A wide variety of biocompatible materials (e.g., silk, gelatin, cellulose, chitosan, alginate, polyurethane, poly (lactide-co-glycolide), polyvinyl alcohol, and poly-ε-caprolactone) can be used [4] in various forms (e.g., nanofiber, woven fabric, film, foam, hydrogel, hydrocolloid, and hydrofiber) for wound dressing fabrication [5]. Silk fibroin (SF), a primary constituent of silk proteins, is one of the most attractive biomaterials. It has excellent biocompatibility and low immunogenicity with good mechanical properties [6]. Furthermore, SF can be readily functionalized on its plentiful functional groups (e.g., carboxyl, amine, hydroxyl, and phenol groups) [7]. Thus, SF-based materials have been studied as promising biomaterials for use as tissue engineering scaffolds and surgical sutures [6]. SF has outstanding performance as a wound dressing material, and accordingly various types of fabrication techniques have been attempted [8]. Electrospinning is an attractive method for SF wound dressing fabrication because electrospun fiber has a large surface area with a high porosity [5,9]. However, the intact electrospun SF nanofiber is limited as a wound dressing material because it can paradoxically provide a preferred environment for infectious bacteria [10].

To prevent bacterial infection and biofilm formation on both skin wounds and dressing material, antibiotics such as penicillin and methicillin have been used. However, the use of traditional antibiotics has been reduced due to an emergence of antibiotic resistant bacteria [11]. Alternatively, other types of antimicrobial materials (e.g., quaternary ammonium compounds [12], silver ions or nanoparticles [13], or antimicrobial polymers [14]) have been tried for wound care. In particular, silver-containing materials present excellent antimicrobial effects [15]. However, these alternative materials have either considerable adverse effect (e.g., cytotoxicity, environmental issues) or low efficacy [12,13,16–18].

In recent years, antimicrobial peptides (AMPs) have received great interest as alternative antimicrobial materials. Generally, AMPs, which exist in mammals, insects, fishes, amphibians, and even some bacteria, play a crucial role in protecting the host from invasive bacteria, fungi, or viruses in conjunction with other immune responses [19,20]. Although AMPs have diverse structures, they share common structural characteristics such as cationic and amphiphilic domains with α-helical conformation. It is believed that such a structure plays a key role in antimicrobial activity by disrupting the bacterial cell membrane [19,20]. AMPs exhibit not only the rapid onset of bacterial killing but also a broad-spectrum of antimicrobial activity with a high efficacy. Furthermore, they do not cause bacterial resistance and therefore are relatively safe in long-term use [21]. For example, the antimicrobial peptide nisin was approved by the Food and Drug Administration (FDA) as a food preservative [22]. In addition, many pharmaceutical companies have been trying to develop AMPs for therapeutic use, and some have been applied in clinical trials [23].

KR12 (KRIVKRIKKWLR) is the shortest antimicrobial motif (residue 18–29) of the human cathelicidin peptide (LL37) [24–26], which is secreted from various human immunocytes and epithelial cells [27]. In addition to antimicrobial activity, LL37 exhibits various bioactivities such as the neutralization of lipopolysaccharides (LPS) [28], the modulation of the inflammatory response [29], and the promotion of re-epithelialization (i.e., migration, proliferation, and the differentiation of epithelial cells) [30–34], which result in an acceleration of the wound healing process [35,36]. In spite of this multi-functionality, the use of LL37 or KR12 is limited because of potential cytotoxicity and susceptibility to proteolysis [37–40]. To reduce its cytotoxicity and increase its antimicrobial stability, AMPs are often immobilized onto surfaces of materials in various biomedical applications such as urinary catheters [41–43], bone or dental implants [44–46], and artificial corneas [47]. Nevertheless, only a few studies have attempted to use AMPs as wound dressing application [48–50], and no study has tested the multi-function of biomaterial-surface-immobilized KR12 peptide.

In this study, we immobilized an antimicrobial peptide (Cys-KR12) onto an electrospun SF nanofiber membranes via a thiol-maleimide coupling method and investigated its antimicrobial activity against different types of pathogenic bacteria with varying immobilization densities. Additionally, the various bioactivities (i.e., proliferation, differentiation, and pro-inflammatory cytokine expression) of the Cys-KR12-immobilized SF nanofiber membrane were evaluated with respect to wound healing.

2. Materials and methods

2.1. Materials

To obtain SF, *Bombyx mori* cocoons were boiled in 0.3% (w/v) sodium oleate and 0.2% (w/v) sodium carbonate solution at 100 °C for 1 h. Degummed cocoons were washed in deionized water and dried. SF solution was obtained by dissolving the degummed cocoons in 9.3 M LiBr (Kanto Chemical) solution at 60 °C for 4 h. The SF solution was then dialyzed against deionized water using cellulose acetate membrane (MWCO: 12–14 kDa) for 3 days, which was followed by freeze-drying. Cys-KR12 (CKRIVKRIKKWLR, analogue-3 of original KR12 from [25], >95% purity) (Fig. S1) was purchased from BeadTech Inc. and all other unspecified chemicals were purchased from Sigma-Aldrich.

2.2. Fabrication of the Cys-KR12-immbolized SF nanofiber membrane

For electrospinning, SF solution was prepared by dissolving regenerated SF sponge in formic acid at 11% (w/v). Then, the dope solution was transferred to a syringe and electrospun onto parchment paper at 13 kV and 0.3 mL/h for 24 h in ambient conditions. After electrospinning, the SF nanofiber membrane was insolubilized by soaking in ethanol for 1 h. Peptide immobilization was conducted by a three-step process with a combination of EDC/NHS chemistry and thiol-maleimide click chemistry (Fig. 1). Briefly, the carboxylic acid groups of SF were activated by N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) (0.4 mg/mL) and N-hydroxysuccinimide (NHS) (0.6 mg/mL) in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.0) for 15 min at room temperature. The carboxyl-activated SF nanofiber membrane was transferred into phosphate buffered saline (PBS) (pH 7.4) and then reacted with N-(2-aminoethyl)maleimide (AEM) linker (0.2 mg/mL) for 2 h at room temperature before washing three times with deionized water. The AEM-conjugated SF nanofiber membrane (SF-AEM) was reacted with various concentrations of Cys-KR12 solution (50, 100, 200, and 500 μg/mL) in PBS for 4 h at room temperature and subsequently washed three times with deionized water. Sample IDs are designated as the concentration of Cys-KR12 solution used in the immobilization (Table 1).

2.3. Surface characterizations

The surface morphology of the SF nanofiber membrane was observed using field-emission scanning electronic microscopy