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Interaction and effectiveness of antimicrobials along with healing-promoting agents in a novel biocellulose wound dressing

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ABSTRACT

An ideal wound dressing should keep the wound moist, allow oxygen permeation, adsorb wound exudate, accelerate re-epithelialization for wound closure, reduce pain and healing time, and prevent infection. Our novel biocellulose-based wound dressing was composed of three components: 1) biocellulose (BC), intended to create a moist and oxygen-permeated environment with exudate adsorption; 2) silk sericin (SS) known for its enhancement of collagen type I production, which is critical for re-epithelialization; and 3) the antiseptic polyhexamethylene biguanide (PHMB). To deliver an effective BC wound dressing, the interactions between the components (PHMB vs. SS) needed to be thoroughly analyzed. In this study, we investigated important parameters such as the loading sequence, loading concentration, and loading amount of the active compounds to ensure that the BC wound dressing could provide both antimicrobial activity and promote collagen production during healing. The loading sequence of SS and PHMB into BC was critical to maintain PHMB antimicrobial activity; silk sericin needed to be loaded before PHMB to avoid any negative impacts. The minimum PHMB concentration was 0.3% w/v for effective elimination of all tested bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, methicillin-resistant *S. aureus*, *Escherichia coli*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*). The amounts of SS and PHMB in BC were optimized to ensure that the dressings released the optimal amounts of both SS to enhance fibroblast collagen production and PHMB for effective antimicrobial activity.

1. Introduction

The ideal wound dressing should promote a suitable environment for wound healing, enable fast tissue regeneration, reduce pain, and prevent infection during the healing process [1]. To accelerate the healing, the wound needs to be moisturized while the excess exudate and toxins must be removed to minimize maceration. In addition, oxygen must be able to permeate through the dressing to supply regenerating cells. Recently, biocellulose (also known as bacterial cellulose) has been developed for several applications, including wound dressing [2,3]. Unlike plant-based cellulose, biocellulose (BC) has an ultrafine network with high porosity which allows a high capability for water uptake [2,4], making it becomes an ideal material for the healing of high exudate wound. The highly uniaxial-oriented nanofibers (3–8 nm) of BC contribute to a high crystallinity content (60–80%) [2], providing impressive mechanical strength for ease of handling in the wet state. Furthermore, BC is considered as an electrostatically neutral material [5], enabling it appropriate for the loading of either positively-charged or negatively-charged bioactive compounds. In addition, the high water retention characteristics of BC create a moist wound healing environment [2,4], which allows for faster healing than a dried environment [6–8].

Up to date, commercial BC wound dressings are available. Some of them are incorporated with drugs or active compounds (i.e., iodine, chlorhexidine, and silver) for antimicrobial purpose. In this study, polyhexamethylene biguanide (PHMB) was chosen as an antiseptic drug to be incorporated in the BC dressing because it displays advantages over other antiseptics [1,9,10]. PHMB (molecular weight 3000 Da) is known as a cationic and strong base which interacts with acids and negatively charged molecules, such as phospholipids found in bacterial membranes [11]. The antimicrobial activity is based on the interaction between PHMB and bacterial phospholipids, which disrupts the integrity of the bacterial membrane, leading to the intrusion of PHMB into the cytoplasm, causing a malfunction in metabolic activity and resulting in the death of the bacterium [12,13]. It is reported that PHMB shows antimicrobial effect against various microorganisms such as yeasts, fungi, and bacteria (both Gram-positive and Gram-negative) with low toxicity in mammalian cells [1,11–13]. PHMB has been loaded into different types of wound dressings, e.g., gauze and bandages [14,15], foam [16,17], nanofibrous membranes of cellulose acetate and polyester urethane

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[18], or BC dressings [10,19,20]. Some of BC dressings loaded with PHMB are commercially available.

To improve the effectiveness of this BC dressing, we here introduce another bioactive factor for an enhancement of the re-epithelialization process which is one of the critical steps in wound healing process. Silk sericin (SS) is chosen for this purpose. SS (molecular weight of 20–310 kDa) is an adhesive protein that binds two fibroin strands in silk fibers [21]. It is a hydrophilic, biocompatible, biodegradable, negatively-charged material [22,23] with various biological properties [24]. It has been used to promote proliferation of human fibroblasts [25] or human epithelial HeLa cells [26] and prevent the UVB-induced apoptosis in human skin keratinocytes [27]. The addition of SS to culture media enhances collagen type I production by the L929 mouse fibroblast cell line [23] and promotes L929 migration [28]. An animal model revealed that SS-treated wounds had reduced inflammatory reactions and faster healing time than Betadine®-treated wounds [29]. In the clinical tests of split-thickness skin wounds, the complete healing time of wounds treated with a SS-releasing dressing was significantly shorter than that required for wounds treated with Bactigras® [30]. Furthermore, SS was shown to enhance re-epithelialization [23,28–33].

To our design, the combination of SS and PHMB into a BC wound dressing should capture the unique benefits of both SS and PHMB. In this study, the interactions between these components that affect the biological properties of BC dressing were investigated. The interaction between PHMB and SS was evaluated by attenuated total reflection Fourier Transform Infrared spectroscopy (ATR-FTIR). The loading quantities and sequence of both compounds were optimized. The antimicrobial test was performed by both the disc diffusion and broth dilution techniques using both Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*, and methicillin-resistant *S. aureus*) and Gram-negative bacteria (*Escherichia coli*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*) to determine minimum bactericidal concentration of PHMB. Then, the SS loading amount was varied to achieve an effective released amount which was suitable for collagen-I production by fibroblast cells. The in vitro release of PHMB and SS from the BC dressing was also evaluated. Finally, the effects of the dual-loaded SS/PHMB released from the BC dressing on the antimicrobial effect were compared with other commercially available antimicrobial wound dressings.

2. Materials and methods

2.1. Materials

Coconut water was obtained from coconuts purchased locally. Ammonium phosphate ($(NH_4)_3PO_4$), glacial acetic acid (CH_3COOH), and other chemicals used were of analytical grade (Sigma-Aldrich, USA). Polyhexamethylene biguanide was kindly provided by Lonza Group Ltd. (Basel, Switzerland). Silk sericin was extracted according to a high temperature and high pressure degumming method described in a previous report [23]. Proteoglycan-IPC, a soluble PG extracted from the nasal cartilage of *Oncorhynchus keta* (Salmonidae), was purchased from Icimaru Pharcos Co., Ltd. (Gifu, Japan).

2.2. Preparation of biocellulose

Biocellulose was prepared from a static culture of an *Acetobacter xylinum* strain (Kasetsart University, Bangkok, Thailand) which was isolated from nata de coco, using a coconut water-based medium. The preparation of the medium was slightly modified from the method developed by Verschuren et al. [34]. One liter of coconut water was boiled and supplemented with 50 g of sucrose, 5 g of $(NH_4)_3PO_4$, and 10 mL of CH_3COOH. The pH of the medium was adjusted to 4.5 with CH_3COOH. Then, 10 mL of *A. xylinum* was added into the pH-adjusted coconut water based medium and poured into molds for fermentation. The fermentation was carried out under sterilized static conditions at 30 °C for 3–5 days to pre-culture the *A. xylinum* strain. After obtaining the stock solution of *A. xylinum*, the fermentation was incubated at 30 °C under static conditions for another 7 days to form the BC. The formed bacterial nanocellulose gels were washed with 2% aqueous NaOH solution at 70 °C for 10 min, and then washed repeatedly until a neutral pH was obtained.

2.3. Preparation of the BC wound dressing containing bioactive compounds

The BC wound dressing was loaded with PHMB and SS. Proteoglycan (PG) which is known as a healing-promoting agent was used as a control for SS. All samples prepared in this study were summarized in Table 1. Each dressing was loaded with either one or two components. In the first study, the BC wound dressing was immersed into PHMB solutions with different concentrations (0.0125–0.6% w/v) for 2 h to determine the minimum bactericidal concentration (MBC) of PHMB loaded into the BC wound dressing.

The second experiment was intended to study the interaction between PHMB and two compounds (SS or PG), the loading sequence of each compound and MBC of PHMB in the dual-loaded BC. The BC wound dressing was immersed in a solution of the first compound for 2 h. Subsequently, the soaked BC was allowed to dry before being soaked in a solution of the second compound for another 2 h. The dual-loaded BC was air-dried before the tests. The PHMB concentration was varied from 0.2–0.4% w/v while the concentration of SS or PG was fixed at 1% w/v.

After the MBC of PHMB was obtained, the loading amount of PHMB was then optimized. In this experiment, SS was first loaded into the BC (the size of BC was $10 \times 10 \times 0.01$ cm^3) before PHMB. The loading amount of SS was fixed at 2 mL of SS solution (1% w/v) per side of the BC wound dressing (or total loading of 4 mL). After 2 h of SS adsorption, PHMB was added into the BC/SS wound dressing; the loading amount ranged from 3 to 6 mL of PHMB solution (0.3% w/v) per side of BC/SS (or total loading was from 6 to 12 mL). Before the tests, the dual-loaded BC was air-dried for 2 h.

Finally, the loading amount of SS on BC was optimized for the effective release amount of sericin. In this experiment, SS was first loaded into BC with different loading amounts, ranging from 2 to 3 mL of SS solution (1% w/v) per side of the BC wound dressing (or total loading of 4 to 6 mL). After 2 h of SS adsorption, the PHMB solution (0.3% w/v) was added to the BC/SS wound dressing at a fixed amount of 5 mL per side of BC/SS or total loading of 10 mL. The dual-loaded BC was air-dried for 2 h before the in vitro release test of SS. In this experiment, the single loaded PHMB on BC was used as a negative control for the SS release experiment.

2.4. Antimicrobial efficacy test

Antimicrobial efficacy of different types of PHMB loaded on BC was evaluated in triplicate by the disc diffusion method (CLSI M2-A9) and the broth dilution method (CLSI M7-A7). Six strains of bacteria were selected for these tests: *B. subtilis* (ATCC 6633, Gram-positive), *S. aureus* (ATCC 25923, Gram-positive), methicillin-resistant *S. aureus* (MRSA, Gram-positive), *E. coli* (ATCC 25922, Gram-negative), *A. baumannii* (ATCC 19606, Gram-negative), and *P. aeruginosa* (ATCC 27853, Gram-negative). Müller Hinton (MH) agar was used for culture, inoculation and antimicrobial efficacy tests. All bacterial strains were cultured on an agar plate at 37 °C for 24 h right before preparation of the inoculum. The inoculum was prepared by selecting three to five isolated colonies of bacteria into 5 mL of Tryptone Soya Broth (TSB), and followed by incubation at 37 °C for 4–6 h. The content of bacteria was verified by a UV/VIS spectrometer (Lambda 25, Perkin Elmer, Waltham, MA, USA) at 625 nm. The absorbance of the inoculum should be between 0.08 and 0.13 for a bacterial content of 1.5×10^8 CFU/mL.

For the disc diffusion method (CLSI M2-A9), one swab was applied on the entire surface of the MH agar plate. Then, the BC containing PHMB ($1 \times 1 \times 0.1$ cm^3) was placed on the MH agar plate and incubated