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Chitosan–aluminum monostearate composite sponge dressing containing asiaticoside for wound healing and angiogenesis promotion in chronic wound

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ABSTRACT

There are many factors that delay healing in chronic wounds including lowering level of growth factors and increasing exudate level comprising high amount of tissue destructive enzymes. Asiaticoside possesses interesting wound healing and angiogenic activities that are employed to stimulate tissue regeneration in wound healing application. This study attempted to develop chitosan–aluminum monostearate (Alst) composite sponge containing asiaticoside for use as an absorbent medical dressing in chronic wound. *N*-methyl-2-pyrrolidone (NMP) was used to enhance homogeneity of asiaticoside in the polymer composite matrix. The sponge dressings were prepared by lyophilization and dehydrothermal treatment (DHT). Functional group interaction, crystallinity, and morphology of the prepared sponges were investigated using FT-IR, PXRD, and SEM, respectively. Physicochemical properties, porosity, hydrophilic/hydrophobic properties and mechanical property, were evaluated. Wound dressing properties, water vapor transmission rate (WVTR), fluid absorbency, oxygen permeation (OP), and bio-adhesive property, were investigated. In vitro asiaticoside release study was conducted using immersion method. Cytotoxicity was studied in normal human dermal fibroblast (NHDF) and normal human epidermal keratinocyte (NHEK). Angiogenic activity of asiaticoside was evaluated using chick-chorioallantoic membrane (CAM) assay. FT-IR and PXRD results revealed the amidation after DHT to enhance the crystallinity of the prepared sponges. The prepared sponges had high porosity comprising high Alst-loaded amount that exhibited more compact structure. Alst enhanced hydrophobicity therefore it reduced the fluid absorption and WVTR together with bio-adhesion of the prepared sponge dressings. Porosity of all sponges was more than 85% therefore resulting in their high OP. Enhancing hydrophobicity of the material by Alst and more homogeneity caused by NMP eventually retarded the asiaticoside release for 7 days. The sponge extractions were non-toxic to the cells moreover they promoted NHDF and NHEK cell proliferation. Asiaticoside and asiaticoside-contained dressings exhibited dose-dependent angiogenic activity in CAM model.

1. Introduction

Chronic wounds are defined as tissue injuries that often reoccur and have not healed within 12 weeks [1]. There are many factors that delayed the chronic wound healing including lowering of growth factors level and high exudate level [2]. Normally, the exudate plays an important role in all stages of wound healing by maintaining moisture and providing nutrient to the wound tissue. However, the exudate in chronic wound is different from acute wound in case of relatively higher level of tissue destructive proteinases which can cause more corrosion to the wound tissue [2]. Therefore, therapeutic approach for chronic wound should be emphasized at tissue regeneration stimulation and wound exudate management.

In case of tissue regeneration stimulation, many researchers have attempted to promote an angiogenesis in chronic wound by delivering the angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic-fibroblast growth factor (bFGF) and/or platelet-derived growth factor (PDGF) to the wound bed [3–7]. This technique offers great potential and exhibits more rapid healing result than the other techniques such as cell or gene delivery, and mechanical stimulation [8]. However, the growth factors have limitations of their poor stability and dramatically high cost.

Natural-derived substances such as asiaticoside from *Centella asiatica* (L.) Urban, resveratrol from *Vitis* spp. and ginsenoside Rg1 from *Panax ginseng* exhibited an interesting angiogenic activity [9,10].

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Asiaticoside is one of triterpene glycosides found in *C. asiatica* (L.) Urban that possessed many interesting biological activities. From aforementioned properties, asiaticoside is interesting to be employed as active compound for chronic wound treatment.

Chitosan possesses many interesting properties to promote wound healing including promotion the fibroblast proliferation, collagen synthesis, integrin engagement and expression of cytokines and growth factors that promote wound healing and angiogenesis as reported in the previous reviews [11–13]. Aluminum monostearate (Alst) is the metal stearate that has been utilized as oil thickening agent in development of muscular injectable implants [14], an excipient of suspensions for prolonged release procaine penicillin and riboflavin [15,16] and an additive of vaccine formulations [17]. For wound healing applications, aluminum stearate was reported to be used as waterproofing agent in skin ointment preparation [18].

In our previous study [19], we successfully developed hydrophobic chitosan–aluminum monostearate (Alst) composite sponges by performing simple sequential steps of mixing, lyophilization and dehydrothermal treatment (DHT) without conducting a neutralization process and using any surfactants or any chemical mediators. This material was stable in aqueous medium and could successfully control the release of asiaticoside with a sustained manner for 2 days therefore it had a potential to be further developed as drug controlled release wound dressing. In this study, we fabricated asiaticoside-loaded chitosan–Alst composite sponge dressings. *N*-methyl-2-pyrrolidone (NMP) was added to the prepared system in order to enhance the homogeneity of asiaticoside in the polymer matrix.

2. Materials and methods

2.1. Materials

Aluminum monostearate (Alst) was purchased from Sigma-Aldrich Co., Missouri, USA. Asiatic acid and asiaticoside (90%) were purchased from Guangxi Changzhou Natural Products Development Co. Ltd., China. Chitosan (97% deacetylation degrees with 70 kDa molecular weight) was purchased from Aqua premier, Chonburi, Thailand. Lactic acid was purchased from Loba Chemie Pyt. Ltd., Mumbai, India. *N*-methyl-2-pyrrolidone (NMP) was purchased from Sigma-Aldrich Co., Missouri, USA. The other reagents were AR grade. Vascular endothelial growth factor ($VEGF_{165}$, human recombinant animal free) was purchased from Merck Millipore, Massachusetts, USA. Fertilized eggs were purchased from Suwanvajokkasikit Animal Research and Development Institute, Kasetsart University, Nakhon Pathom, Thailand. Normal human dermal fibroblast (NHDF) (passage numbers 4–12) and normal human epidermal keratinocyte (NHEK) (passage numbers 3–6) were supported by the Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan. Cell count reagent SF (WST-8) was purchased from Nacalai Tesque, Kyoto, Japan. Dulbecco's modified Eagle's medium (DMEM) (Complete) was purchased from Gibco, Invitrogen K.K., Tokyo, Japan. Fibroblast basal medium (FBM) and keratinocyte basal medium (KBM) were purchased from Lonza Group Ltd., Tokyo, Japan.

2.2. Methods

2.2.1. Sponge dressing preparation

Briefly, 4% w/w chitosan solution (CL) was prepared by dissolving chitosan in 2% w/v lactic acid solution with continuous agitation until the clear solution was obtained. Asiaticoside was completely dissolved in NMP prior to mixing with the CL solution. NMP and asiaticoside were added into the solution at concentration of 2.5% and 0.012% w/w (1200 μg/g), respectively. In case of Alst-contained dressing, various amounts of Alst (0.5, 2.5, or 5.0% w/w) were added into the previous mixture and then homogenized at 8000 Hz for 5 min using homogenizer (IKA®T25 Digital Ultra-Turrax, IKA Works (Asia), Malaysia) in order to disperse Alst in the aqueous mixture homogeneously. Subsequently, the mixtures were stirred for another 24 h using a magnetic stirrer at 1000 rpm. Thereafter, the obtained mixtures were fabricated by using a lyophilization technique as the following details. Approximately 20 mL of the mixture was filled in an aluminum cup (8 cm-diameter). The cup was then sealed with an aluminum foil and surrounded the lateral side with a cotton fabric as a thermal insulator prior to be frozen at −20 °C for 24 h and dried employing a freeze dryer (Triad, Labconco, Missouri, USA) for 72 h. The lyophilized sponge dressings were then treated by dehydrothermal treatment (DHT) at 110 °C for 24 h in a vacuum oven in order to stabilize the sponge structure in an aqueous medium. The sponge dressing containing 0%, 0.5%, 2.5% and 5.0% w/w Alst was named as CD, CD05, CD25 and CD50, respectively.

2.2.2. Physicochemical properties characterization

Functional group interaction of the prepared sponge dressings was evaluated using an FT-IR spectrophotometer (Nicolet 4700, Becthai, USA) in a wavenumber range of 4000–400 cm^{-1} using a KBr pellet method for the solid substances, and liquid detector accessory (smart multi-bounce HATR) for the liquid substances. Crystallinity of the raw materials and the prepared dressings was characterized using PXRD (Miniflex II, Rigaku Corp. Tokyo, Japan) in a 2θ range of 4–60°. Powder of the raw materials was compressed into the 2 mm-depth sample holder before test whereas the sponge dressings were cut (2×2 cm^2) and adhered to the sample holder using double adhesive tape. The test was performed using 30 kV voltages and 15 mA currents. The Cu-Kα was used as the X-ray source which liberated the x-radiation of 1.541841 Å wavelength (λ). Morphology of the surface, bottom and cross-sectioned of the prepared dressings was observed under SEM (Maxim 200 Cam scan, Cambridge, England) at 50 times magnification in a secondary electron image (SEI) mode.

2.2.3. Porosity

Porosity (ε) of the chitosan sponges was measured using liquid displacement method as described previously [20] with some modifications. Ethanol was employed as the liquid in the experiment. A 20 mL-ethanol-contained-measuring cylinder was accurately weighed (M1). The dried sponge was weighed (Ms) and then pre-immersed in ethanol in a glass bottle prior to be sonicated in water bath at 30 °C for 1 h in order to assist complete penetration of ethanol into the sponge pore. Thereafter, the ethanol-impregnated sponge was then transferred into the 20 mL-ethanol-contained cylinder. Ethanol volume in the cylinder was then readjusted to 20 mL before weighing (M2). Subsequently, the sponge was removed from the cylinder, and the rest was weighed (M3). Porosity of the sponge was calculated ($n = 3$) using Eq. (1). Where Vp is volume of the sponge's pore and Vs is volume of the sponge's skeleton.

$$\text{Porosity } (\varepsilon) = Vp/(Vp + Vs) = (M2-M3-Ms)/(M1-M3). \quad (1)$$

2.2.4. Mechanical strength

Mechanical strength of the prepared sponges was evaluated using a texture analyzer (Charpa Techcenter, Godalming, Stable Micro Systems Ltd., UK) in compression mode. The sponge was cut into square shape with 1×1 cm^2 size and approximately 3.5 mm thickness. The instrument was set up with 5 kg load cell. The cylindrical probe (3 mm-diameter (P/3), 7.07 mm^2 contact area, stainless) was set at 10 mm away from the base. Sample was placed on the base by fixing their center to the probe. Test speed was 2 mm/s and a target was 6% strain. Stress–strain curve was plotted by the Exponent® software. The highest stress value was recorded ($n = 6$).

2.2.5. Hydrophilic/hydrophobic property evaluation

Hydrophilic/hydrophobic property of the prepared sponges was evaluated using goniometer (FTA 1000, First Ten Angstroms, USA)