Novel Wound Dressing of Non-woven Fabric Coated with Genipin-crosslinked Chitosan and *Bletilla Striata* Herbal Extract

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Abstract

This study proposes a novel design of an easily stripped bi-layered composite that comprises of an upper layer of soybean protein non-woven fabric, coated with a lower layer, genipin-crosslinked chitosan and *bletilla striata* herbal extract (GCB) film, as a wound dressing material (GCB/NWF). Preliminary tests revealed the *in vitro* characteristics of the genipin-crosslinked chitosan (GC) film. Fourier Transform Infrared Spectroscopy (FTIR) has revealed that secondary amide linkages are formed by the reaction between the genipin ester group and the chitosan amino group, yielding a polymeric network structure. Genipin and chitosan were released from the soaked GC film. A cytotoxic test revealed that 20 ppm of the genipin in the culture medium is the concentration threshold, over which it is cytotoxic to production of L929 fibroblasts. However, the chitosan and *bletilla striata* herbal extract not only were not toxic but also promoted the viability and growth of L929 fibroblasts. Additionally, the GCB film effectively supported cell attachment and growth. The result of the *in vivo* histological assessment revealed that epithelialization and reconstruction of the wound were achieved by covering it with the GCB/NWF wound dressing material, which was easily stripped from the wound surface without damaging the newly regenerated tissue.

Keywords: Chitosan, Genipin, Cross-linking, *Bletilla striata*, Non-woven fabric

1. Introduction

Most recent progress in wound management has been based on physiological support. As infections delay healing and increase scar formation, achieving closure as soon as possible is important. An effective wound dressing typically has the following properties: the dressing material must be able to absorb any bodily fluids exuded from the wounded area. The material should permit evaporation of moisture at a certain rate. It should not excessively adhere to the surrounding tissue, so that it can be easily removed after healing. If removal of the dressing is not correctly performed, additional damage to the wound may prolong recovery. An easily stripped wound dressing material must be developed to reduce the pain suffered by patients when wound dressings are frequently changed. This study proposes a novel bi-layered composite as a wound dressing material. The upper layer of the composite is a non-woven fabric that prevents wound infection, wound surface dehydration, and bacterial invasion, while allowing for drainage of the wound exudates. The lower layer of the composite comprises genipin-crosslinked chitosan and *bletilla striata* herbal extract (GCB) film that is in contact with the wound, which has a high adsorption capacity for fluids, and promotes tissue reconstruction.

Owing to its advantages of having a large surface area, high porosity, the absence of dust, ventilation and ease of processing, non-woven cloth is used extensively in applications that range from medical dressings to everyday cleaning products. This study has developed a novel non-woven wound dressing that is composed of soybean protein fibers. Soybean protein fiber is a renewable botanic

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protein fiber with fine monofilaments, low density, high strength and excellent acid and alkali resistance [1]. Soybean protein fiber has not only the tactile feel of natural silk but also the mechanical strength of synthetic fibers. The material is biocompatible with human skin. The fiber also contains numerous amino acids that are required by the human body and the preservation of health. Chitosan has been studied for use in many other biomedical applications, including drug delivery carriers, surgical thread, bone-healing materials and particularly wound dressings [2-4]. As a functional material, chitosan has special characteristics and advantages, including biocompatibility, biodegradability, haemostatic activity, antibacterial properties, low cost and the ability to accelerate wound healing [5-7]. Moreover, the free amino groups in chitosan can be chemically derivatized under mild reaction conditions to promote biological activity or to modify its mechanical properties. The rapid degradation of chitosan reduces its utility as a scaffold in tissue regeneration and drug-delivery applications. Various processes have been proposed to reinforce the structural stability of chitosan in acidic solutions using cross-linking treatments [8-11]. Genipin and its related iridoid glucosides extracted from the fruits of *Gardenia jasminoides Ellis* have been extensively used as an antiphlogistic and cholagogue in herbal medicine [12]. Furthermore, genipin can spontaneously react with amino acid or proteins to form dark blue pigments that are used to fabricate food dyes [13]. Genipin has been used to cross-link functional amine groups present in natural tissues and native polymers with many fewer cytotoxic effects than that of the cross-linking agent glutaraldehyde [14-16]. The experimental results in these studies motivated this study of the feasibility of the use of genipin in biodegradable chitosan films for wound-dressing applications.

Traditional Chinese medicine (TCM) has a long history and provides unique advantages in wound healing. It can be used to provide a favorable micro-environment for the wound healing, prevent inflammation, and reduce the syndromes that may affect a wound with a sterilized dressing. Medical practice over a long period of time has demonstrated that TCM is highly biocompatible when it is in contact and interacts with human body tissues, body fluid or blood. Hence, TCM can be exploited with biological materials to improve the physicochemical properties and function of dressing material. Given an effective biodegradation delivery system, added TCM could accelerate wound healing. The traditional Chinese medicine *bletilla striata* has been used extensively to treat alimentary canal mucosal damage, ulcers, bleeding, bruises and burns. *Chinese Pharmacopoeia* states that *B. striata* supports functions of hemostasia and detumescence and promotes recovery [17]. Accordingly, this study proposes a novel design of an easily stripped bi-layered composite that comprises an upper layer of soybean protein non-woven fabric that is coated with a lower layer, genipin-crosslinked chitosan and *bletilla striata* herbal extract (GCB) film as a wound-dressing material (GCB/NWF). Preliminary tests revealed the *in vitro* characteristics of the genipin-crosslinked chitosan (GC) film. The cross-linking reaction between genipin and chitosan was identified by FTIR spectra and the substances that were released from the soaked GC film were analyzed by the UV-vis. Furthermore, the biological activity of the substances (genipin and chitosan and *bletilla striata* herbal extract) or GCB film co-cultured with L929 fibroblasts was studied *in vitro*. Finally, an *in vivo* experiment was performed to elucidate wounds that had been treated with the dressing material in a rat model. The progress of wound repair towards healing with the GCB/NWF dressing material was assessed histologically.

2. Materials and methods

2.1 Preparation of materials

2.1.1 Bletilla striata herbal extract

The Chinese medicine *bletilla striata* was obtained from a local Chinese medicine store (Xing Long Pharmaceutical Co., Ltd, Taichung, Taiwan, ROC) in dry form. Preparation procedures for *bletilla striata* herbal extract were standardized. Briefly, a 40 g ground specimen of *bletilla striata* was homogenized and dispersed in 80°C double-distilled water (dd water) for 4 h. The aqueous extracts were filtered to remove impurities and concentrated at 40°C using vacuum evaporation. The condensates were then freeze-dried to obtain the final powder used in this experiment. The identity of *bletilla striata* was confirmed by experts in pharmacognosis.

2.1.2 GC and GCB films

Chitosan, M_w ~70,000 and > 85% deacetylated, was purchased from VA & G Bioscience, Inc. (Taoyuan, Taiwan). A chitosan aqueous solution of 3% (wt/v) was prepared by dissolving chitosan powder in distilled water that contained 1% (v/v) acetic acid. Genipin was obtained from Challenge Bioproducts Co. (Taichung, Taiwan). Twenty ml chitosan solutions were then mixed with different volumes of 20% (wt/v) genipin solutions to yield the final genipin-chitosan mixtures with various weight percentages of genipin solutions. After vigorous stirring for 2 min, the mixture began to turn light bluish and became increasingly viscous. It was then immediately cast on glass plates and dried to constant weight at ambient temperature. The cross-linked chitosan became dark bluish after one day. The cross-linked chitosan films prepared with weight percentages of genipin solution from 0.01 to 0.1 wt.-% were labeled from GC 10 to GC 100, respectively. The uncross-linked (pure) chitosan film was labeled as GC 00. The dried material was then washed with aqueous 1 N NaOH to remove residual acid, and finally thoroughly rinsed with ultra-pure water. The GCB film was prepared using a similar procedure to that for preparing the GC film. The mixtures of chitosan and *bletilla striata* herbal extract were prepared by mixing 20 ml of 3% (wt/v) chitosan solution with 6 ml of 20% (wt/v) *bletilla striata* herbal extract. The genipin solution was then added to the chitosan-*bletilla striata* herbal extract mixtures to prepare GCB film.

2.1.3 A bi-layered GCB/NWF wound dressing material

One kilogram of soybean protein fibers, purchased from

Dandy Company (Sanchong City, Taiwan), was combed using a nonwoven-comber under many cylinders and doffers of roller cards to produce a fiber web. Then, a thick regular fiber web was formed under a vertical web layer in a continuous process. Finally, the soybean protein non-woven fabric (NWF), was prepared and needling was used to promote the coalescence of fibers. The aforementioned genipin-chitosan-*bletilla striata* herbal extract mixtures were prepared. Subsequently, the soybean protein NWF (4 × 4 cm in square) was placed onto the surface of the mixtures. The mixtures were air-dried at room temperature for 24 h and frozen at -80ºC for 24 h, before being dried in a freeze-drying vacuum oven to yield a bi-layered wound dressing material that consists of a GCB film and soybean protein NWF.

2.2 Characterization of GC films

2.2.1 FTIR spectrometric analysis

Since genipin was used as a cross-linker to prepare the cross-linked chitosan film, the cross-linking reaction between genipin and chitosan was identified by FTIR spectra. FTIR analyses of the uncross-linked and cross-linked chitosan films with approximately the same thickness were conducted using a Jasco system 4100 FTIR spectrometer (Jasco, Tokyo, Japan). The spectra were obtained over the range of 4,000–1,000 cm^{-1}. Solvents of each sample were evaporated and dried in a vacuum oven to prepare them for FTIR analysis.

2.2.2 Measuring genipin and chitosan release from GC film

The sterilized GC film was placed in a capped plastic tube that was filled with 5 ml deionized distilled water, and was maintained in an incubator at 37ºC. At predetermined times, the soaking solution was collected to measure the optical density (OD value) of the released genipin and chitosan using an ultraviolet-visible spectrophotometer at 238 nm and 230 nm, respectively. The standard OD value-to-concentration curves were based on the OD value of serial concentrations of genipin and chitosan solutions, respectively.

2.3 In vitro cell culture

2.3.1 Cultivation of L929 fibroblasts

Cell culture experiments were performed using mouse L929 fibroblasts (BCRC No. 60091, Bioresources Collection and Research Center, Hsinchu, Taiwan). Cells were seeded in plastic culture dishes and cultured in Dulbecco's Modified Eagle Medium (DMEM, containing 4500 mg/L D-glucose, 2 mM L-glutamine and 110 mg sodium pyruvate, Gibco Invitrogen) that was supplemented with 10% fetal bovine serum (Gibco Invitrogen) and 1% (v/v) antibiotics (10,000 U/ml penicillin G, 10 mg/ml streptomycin and 0.025 mg/ml amphotericin B, Biological). The medium was renewed every three days and cultures were maintained in a 5% CO_2 incubator at 37°C. After about 80% confluence was reached, the cells were detached using 0.25% trypsin-EDTA (Gibco Invitrogen).

2.3.2 Indirect cytocompatibility study

The cultured L929 fibroblasts were seeded in a 96-well plate at a cell density of 5×10^3 per well and incubated for 24 h. The culture medium was replaced with mixed solutions of various concentrations of genipin or chitosan, or *bletilla striata* herbal extract, and new culture medium, in a volume ratio of 1:1. In the control group, PBS was mixed with the culture medium in the same ratio (1:1) for use in cell cultures. After 48 h of culturing, the viability of cells in these culture mediums was evaluated by MTT assay. In this study, the number of viable cells was calculated by converting the optical density (OD) values of MTT assay into cell numbers according to the standard curve.

2.3.3 Direct cytocompatibility study

Test sample GCB film and uncoated glass slide as a negative control were placed on the bottom of 24-well tissue culture plates. The specimens of each sample were sterilized in 70% ethanol and then washed in culture medium before the cell culture procedure was performed. One ml of cell suspension with a density of 5×10^3 cells/ml was seeded uniformly on the surface of each test sample and maintained in a CO_2-controlled incubator at 37ºC. After incubation, cellular constructs were harvested, rinsed twice in PBS to remove non-adherent cells and then fixed with 2.5% glutaldehyde at 4ºC overnight. Scanning electron microscopy was used to visualize the manner of cell attachment and the degree of cell spreading on the test samples after culturing for 24 h. The morphology of the cells that adhered to the test samples was also observed by light microscopy (Zeiss) at a magnification of 100× after culturing for 48 h.

2.4 Animal study

Twenty Wistar rats weighing approximately 400–500 g were anaesthetized with pentobarbital prior to testing. Rat dorsal hair was removed using an electric razor. One full-thickness 4 × 4 cm^2 wound was created by dermoepidermic excisions on each rat. The wound was covered with an equal size of the GCB/NWF composites (n=10) or non-woven gauze pad as a comparison (n=10) and weekly changed. Animals were housed in individual cages. At postoperative days 7 and 14, macroscopic observations of each wound were made. Additionally, specimens taken from the wounds and surrounding skin were fixed with 10% phosphate-buffered formalin and stained with hematoxylin-eosin (HE) reagent for histological examinations.

2.5 Statistical analysis

All measurements were made in triplicate and expressed as means ± standard deviations. Single factor analysis of variance (ANOVA) was employed to evaluate the statistical significance of the results. It was followed by the post hoc analysis of SIDAK. Statistical significance was associated with a probability $P < 0.05$.

3. Results

3.1 Characterization of GC films

3.1.1 Reaction between genipin and chitosan

The reaction between genipin and chitosan proceeded at a moderate rate after the two solutions were mixed, and was monitored by detecting the changes in the physical appearance of the mixtures. Figure 1 presents a photograph of pure chitosan (GC 00) and genipin-crosslinked chitosan (GC 10, 15, 25, 50 and 100) films. The thicknesses of the dried films were measured to be 0.6 ± 0.032 mm. The pure chitosan film was yellowish, while the genipin-crosslinked chitosan films were dark bluish. Also, the extent of the color change due to the reaction depended on the genipin concentration. However, a color change of similar degree was observed between GC 25 and GC 100 films as the genipin concentrations were increased over 0.025 wt.-%. Figure 2 shows the FTIR spectra of chitosan before and after treatment with genipin. The absorbance at 1,570 cm^{-1}, corresponding to primary amine groups, decreased during upon genipin treatment because these functional groups were consumed during the reaction. Additionally, an increase in the amide peak near 1,640 cm^{-1} was observed, indicating the formation of secondary amides in the reaction between the ester groups on genipin and the amino groups on chitosan.

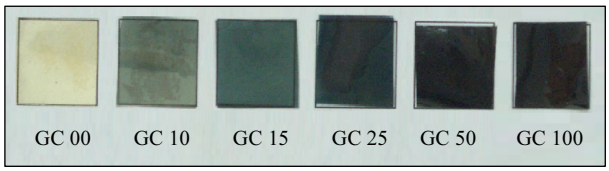


Figure 1. Photograph of pure chitosan and genipin-crosslinked chitosan films.

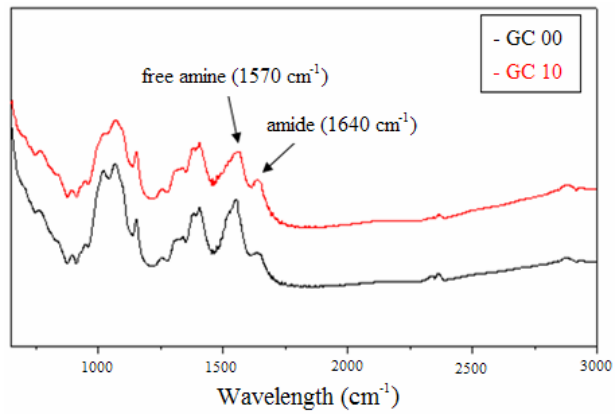


Figure 2. FTIR spectra of chitosan before and after treatment with genipin.

3.1.2 Genipin and chitosan released from GC films

In our earlier work [18], based on the fixation index, the measured swelling ratios and thermal properties, the minimum content of genipin required to crosslink the chitosan fully was 0.025 wt.-%. Therefore, Fig. 3 plots the concentration curves of the genipin and chitosan released from the GC 25 film in soaking solutions. During the first seven days, the GC film released genipin and chitosan rapidly. The early release pattern of genipin and gelatin indicated that uncross-linked genipin and chitosan remained in the GC film as the material was being prepared. Therefore, the uncross-linked genipin and chitosan molecules in the GC film were released immediately after the film was soaked. During the soaking period of seven to 28 days, the concentrations of genipin and chitosan that were released from the GC film increased slowly. Also, the release of genipin and chitosan was attenuated because most of each remained in the cross-linked network structure of the GC film. In contrast, the rates of release of genipin and chitosan increased at an accelerating rate after 28 days of soaking. The results indicated that the genipin and chitosan molecules were dissolved in and continuously released into the soaking solution. As the cross-linked network structure of the GC film was degraded to a certain extent, the release rates of genipin and chitosan increased.

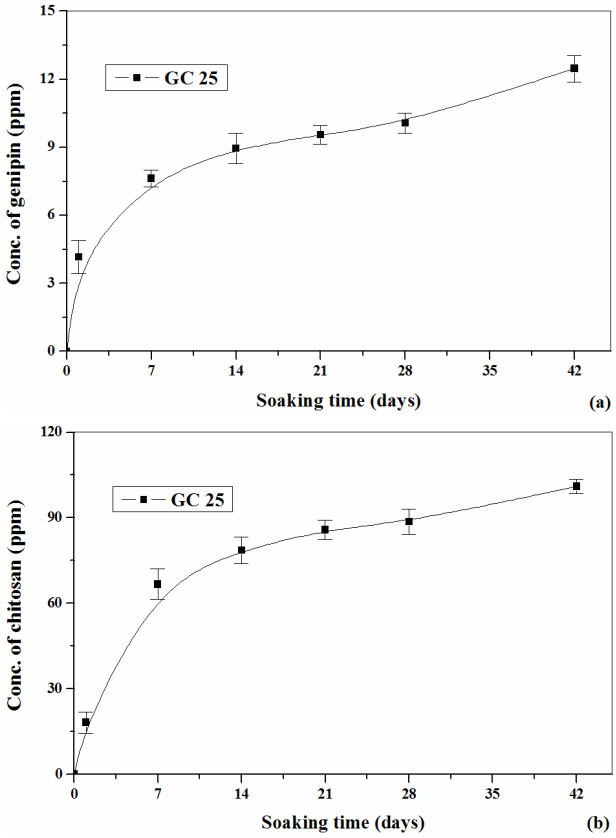


Figure 3. Concentration of (a) genipin and (b) chitosan released in soaking solutions after genipin-crosslinked chitosan film were soaked in deionized distilled water during soaking period.

3.2 In vitro cell culture

3.2.1 Effects of genipin, chitosan and bletilla striata herbal extract on L929 fibroblasts culture

Figure 4 plots the effect of genipin concentration on the number of cells after culturing for two days. After two days of culture, the MTT assay showed the number of cells in the

control group remained approximately 1.38×10^4. In the experimental group, the L929 fibroblasts were separately cultured with media that contained 5, 10, 15 and 20 ppm of genipin for 48 h. When the genipin concentrations exceeded 20 ppm, the number of the L929 fibroblast cells were significantly decreased and lower than those in the controls ($P < 0.05$). Figure 5 shows the effect of chitosan concentration on L929 fibroblasts after culturing for 48 h. The cells proliferated from 1.39×10^4 to 1.42×10^4 as the chitosan concentrations increased from 50 to 200 ppm. The results suggest that the chitosan in this concentration range promoted the growth and proliferation of L929 fibroblasts. Figure 6 presents the effect of *bletilla striata* herbal extract on L929 fibroblasts after culturing for 48 h. The results reveal that the number of cells remained around 1.1×10^4 in the control group. However, the number of cells in the experimental groups increased with the concentrations of *bletilla striata* herbal extract from 20 to 60 ppm. When *bletilla striata* herbal extract concentrations exceeded 60 ppm, the effect of *bletilla striata* herbal extract on L929 fibroblasts proliferation reached a plateau. The results indicate that the *bletilla striata* herbal extract promoted the proliferation of L929 fibroblasts.

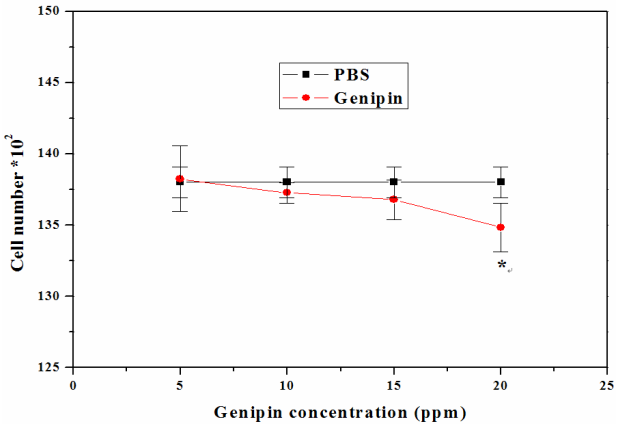


Figure 4. Effect of genipin concentration on the number of cells after culturing for 48 h. *Groups that differed significantly from control (* $P < 0.05$).

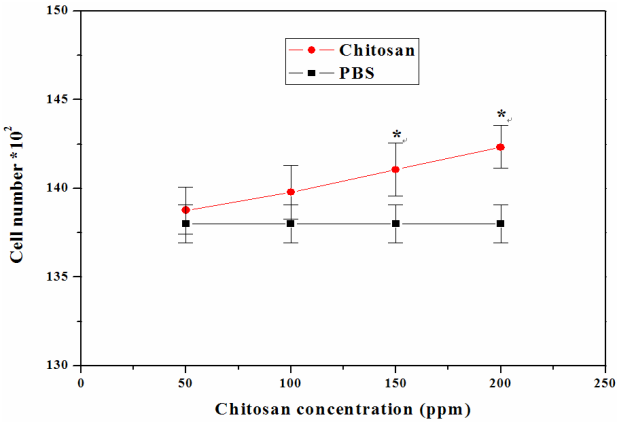


Figure 5. Effect of chitosan concentration on the number of cells after culturing for 48 h. *Groups that differed significantly from control (* $P < 0.05$).

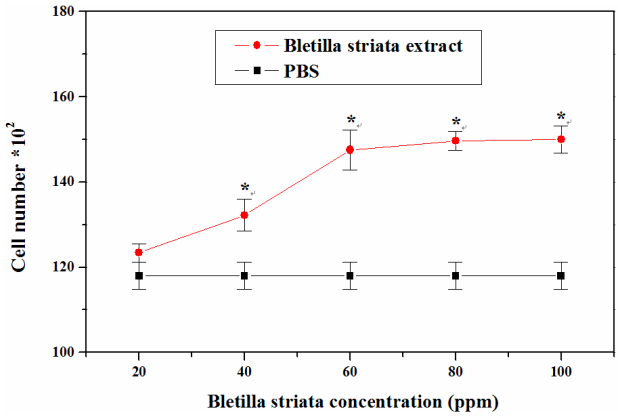


Figure 6. Effect of *bletilla striata* herbal extract on the number of cells after culturing for 48 h. *Groups that differed significantly from control (* $P < 0.05$).

3.2.2 L929 fibroblasts cultured with GCB film

The initial cell attachment and spreading of cells were observed under a scanning electron microscope. Figure 7 shows SEM micrographs of L929 fibroblasts that had been cultured on the surfaces of an uncoated glass slide and the GCB film after 24 h of culturing. Most cells that were cultured on the uncoated glass slide remained round or oval, revealing that they had come into weak contact with the surface of the glass slide (Fig. 7a). Cells on the surface of the GCB film exhibited a spindle-shaped morphology, and the originally round cells became spread out shape with polygonal morphology (Fig. 7b). These results suggest that L929 fibroblasts preferentially attached to the surface of the GCB film. Under high magnification, cells on the surface of the uncoated glass slide had a spherical and compact morphology with smooth dorsal surfaces and short cytoplasmatic processes (filopodia), as shown in Fig. 7c. However, on the surface of the GCB film, cells were polygonal with rough dorsal surfaces, long cytoplasmatic filopodia and ruffled surfaces (Fig. 7d), indicating that they had adapted effectively as they migrated on the GCB surfaces.

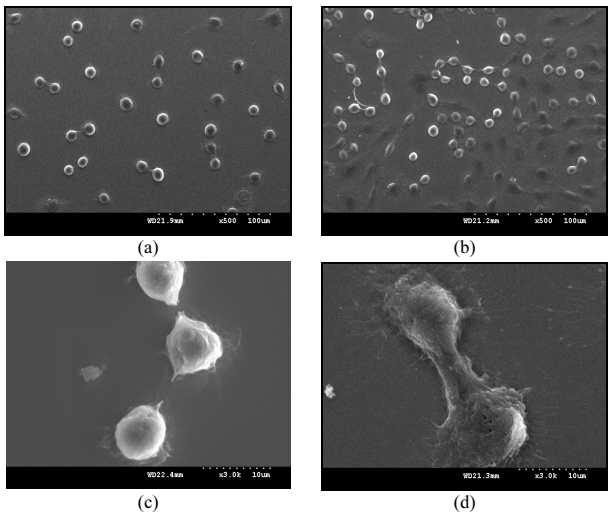
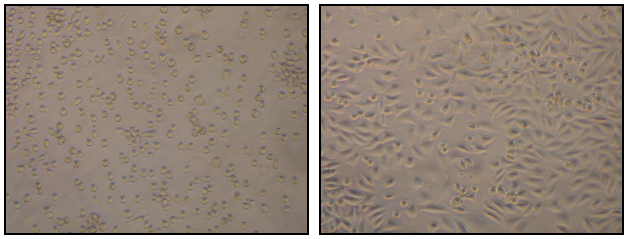


Figure 7. SEM micrographs of L929 fibroblasts cultured on surfaces of (a) an uncoated glass slide (500×); (b) the GCB film (500×); (c) an uncoated glass slide (3K×) and (d) the GCB film (3K×) after a 24 h culture.

Figure 8 presents optical photomicrographs of L929 fibroblasts that were attached to and grew on the surfaces of an uncoated glass slide and the GCB film after a 48-hr culture. These figures indicate that both the shapes and the number of the attached cells varied with the substrate. Most cells that were attached to the surface of the uncoated glass slide (Fig. 8a) remained spherical, without extensive spreading or evidence of much activity. However, on the surfaces of the GCB film (Fig. 8b), most cells changed from their original spherical shape to elongated or spindle-like. The results demonstrate that all cells were extensively spread over the surface and had the typical morphology of fibroblasts during a prolonged cell culture, suggesting that the GCB film supported cell attachment and growth, with no toxic effect on the attached cells.

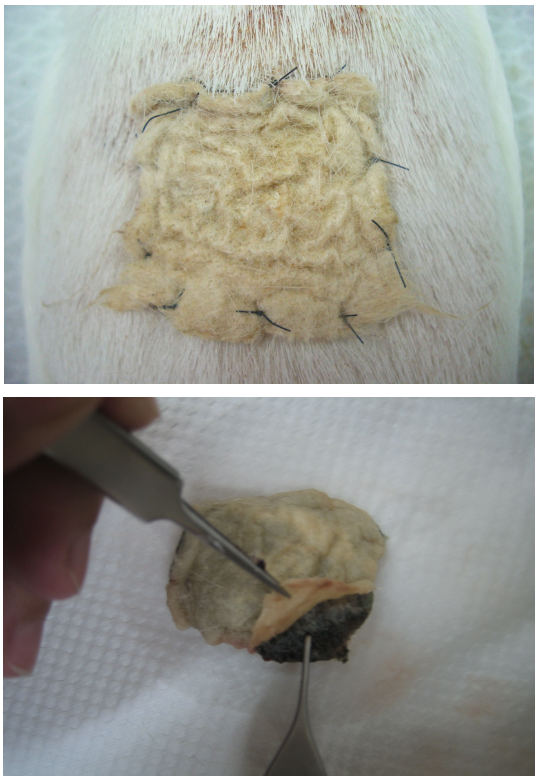


(a) (b)

Figure 8. Light micrographs of L929 fibroblasts cultured on the surfaces of (a) an uncoated glass slide and (b) the GCB film after a 48 h culture. Original magnification: 100×.

3.3 Wound healing

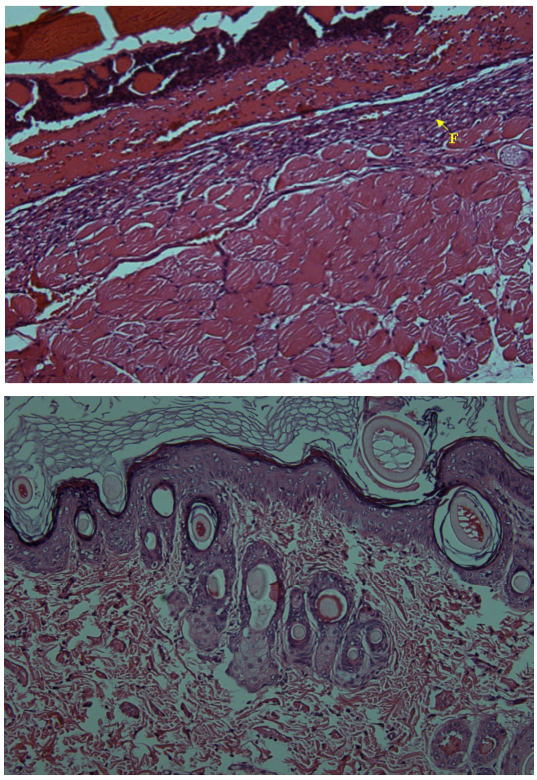
No test sample was infected during the investigation. On postoperative day 7, the GCB/NWF wound dressing material adhered uniformly to the excised wound surface and adsorbed the wound exudates, as shown in Fig. 9a. When the GCB/NWF wound dressing material was removed, the non-woven fabric was easily stripped from the wound surface without damaging newly regenerated tissue, as presented in Fig. 9b. Histological examinations of the wounds that had been covered with the GCB/NWF wound dressing material showed that although the epithelialization process was evident, many of the fibroblast fibers were present in the dermis, as presented in Fig. 10a. On postoperative day 14, the GCB/NWF-dressed wound was epithelialized. As shown in Fig. 10b, this epithelium was well organized and covered with a horny layer; the dermis contained many fibroblasts and collagen fibers. The newly synthesized collagen in the wound was well organized and all oriented along a single axis parallel to the skin surface, indicating perfect regeneration of the damaged tissue. Figure 11 shows a macroscopic photograph and histological examination of the wounds that had been treated with the non-woven gauze pad, when it was removed on postoperative days 7 and 14. The dermis was also rich in fibroblasts and mesenchymal progenitor cells in the contrast group on day 7; however, no complete epidermis had formed (Fig. 11a). On postoperative day 14, the dermis was inflamed, as presented in Fig. 11b, because the weekly removal of the dressing damaged newly regenerated epidermal tissue. Conversely, the removal of the GCB/NWF wound dressing material did not damage any of the newly regenerated tissue.



(a)

(b)

Figure 9. Macroscopic photographs of the GCB/NWF-dressed wound on postoperative day 7. (a) The GCB/NWF composite had adhered uniformly to the excised wound surface and (b) the non-woven fabric was easily stripped from the wound surface.



(a)

(b)

Figure 10. Histological examinations of the wounds that had been treated with the GCB/NWF wound dressing material (200 × magnification) on postoperative (a) day 7 and (b) day 14.

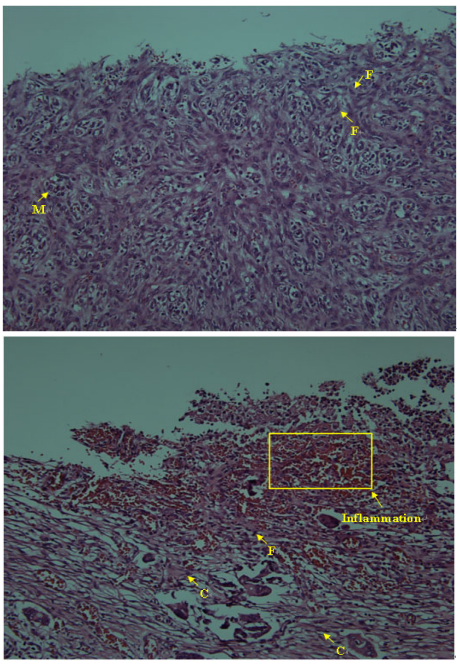


Figure 11. Histological examinations of the wounds that had been treated with the non-woven gauze pad (200 × magnification) on postoperative (a) day 7 and (b) day 14. (C: collagen fibers; F: fibroblast fibers; M: mesenchymal progenitor cells)

4. Discussion

Genipin is known to react exclusively with primary amines [13]. Accordingly, the dark bluish color of the genipin-chitosan mixtures was probably produced by the reaction of genipin with the amino groups of chitosan. Chitosan cross-linking was observed as the color changed from transparent to dark blue within several hours, following treatment with genipin, as shown in Fig. 1. Touyama et al. [13] and Djerassi et al. [19] reported that the blue pigmentation is caused by a spontaneous reaction between genipin and amino acids (primary amines). The cross-linking reaction between the genipin ester group and the chitosan amino group formed secondary amide linkages, yielding the polymeric network structure, as shown in Fig. 2 [20]. Hence, genipin can form intra- and inter-molecularly cross-linked products with heterocyclic structures.

The soaked GC film released genipin and chitosan. Notably, three distinct release stages were observed during the soaking period of the GC films, as shown in Fig. 3. The cytotoxic test implied that 20 ppm was the concentration threshold above which the genipin in the culture medium was cytotoxic toward L929 fibroblasts, as shown in Fig. 4. Fortunately, the genipin concentrations released from the GC films in the soaking solutions were all lower than 15 ppm, as shown in Fig. 3a. Therefore, the GC films used in this study are probably not cytotoxic. As presented in Fig. 3b, the concentration of chitosan released from GC films was approximately 100 ppm after 42 days of soaking, activating the L929 fibroblasts and promoting their growth and proliferation, as shown in Fig. 5. The mechanism by which chitosan interacts with fibroblast cells is unknown, but chitosan has been reported to bind serum factors, such as growth factors, perhaps protecting them from degradation or exhibiting activating effects that would further stimulate cell proliferation [21]. However, polycationic chitosan preferentially interacts with the negatively charged cell surface. These factors influence cell adhesion, cellular bioactivity [22,23], tissue remodeling and ultimately the quality of the regenerated tissue. Chitosan has also been found to interact with epidermal cells, and appears to stimulate re-epithelialization in experimental wounds in dogs and rats.

Figure 6 indicates that the *bletilla striata* herbal extract promoted the proliferation of L929 fibroblasts. *Bletilla striata* has been employed medicinally for over 1500 years in traditional Chinese medicine. The root (actually a pseudobulb) exhibits antibacterial, anti-inflammatory, antiphlogistic and demulcent properties. Wang et al. purified and characterized *Bletilla striata* polysaccharide (BSP), isolated from the herb *bletilla striata* [24]. Their study showed that BSP induced vascular endothelial cell (EC) proliferation and vascular endothelial growth factor (VEGF) expression, both of which should promote wound healing. The spreading of the cells and the presence of filopodia on the cell membranes was evident when these cells were activated on the surface of GCB film, as shown in Fig. 7. Additionally, the mimic extracellular matrix, excreted by the cells, was observed on the surface of the GCB film, suggesting that the GCB film is a more favorable surface for cell growth than that of an uncoated glass slide. The number of attached cells also varied slightly with the substrate, as presented in Fig. 8. The comparative results indicate that the adhesion and spreading of cells were greater on the surface of the GCB film than on the uncoated glass slide.

Both chitosan and soybean protein non-woven fabrics are hydrophilic. In our earlier study [18], water contact angle analysis indicated that cross-linking reduced the hydrophilicity of the chitosan film. Therefore, the non-woven fabric was then placed onto the surface of a chitosan solution or a genipin-chitosan mixture. Once the non-woven fabric had been placed on the surface of the chitosan solution, the hydrophilic uncross-linked chitosan solution infiltrated the non-woven fabric, and the more viscous cross-linked chitosan solution adhered to the surface of the non-woven fabric. Accordingly, a complex entangled structure was formed between the uncross-linked chitosan layers and the non-woven fabric. However, the non-woven fabric was separated from the cross-linked chitosan layers to produce a bi-layered structure. Reducing the complex entanglement of chitosan layers with soybean protein fibers promotes stripping from the wound surface without damage to the newly regenerated tissue in the animal study, as presented in Fig. 9. In the *in vivo* study, wounds were observed 7 days after injury. The GCB/NWF wound dressing material adhered uniformly to the wound surface without any fluid accumulation. The main goal of this work was to develop a bi-layered wound dressing material that could be easily stripped from the wound surface without damaging the newly regenerated tissue. As stated above, cross-linking reduces the hydrophilicity of chitosan films. Hence, a GCB film was adopted to solve the problem of

entanglement. Animal test results show that the application of GCB/NWF wound dressing material reveals perfectly regenerated damaged tissue and solves the problem of re-injuring the wound when the dressing material is changed, revealing that it is an effective wound dressing material.

5. Conclusions

This study has proposed a novel design of an easily stripped bi-layered composite of an upper layer of soybean protein non-woven fabric that is coated with a lower layer, genipin-crosslinked chitosan (GC) and *bletilla striata* herbal extract (GCB) film as a wound dressing material (GCB/NWF). FTIR results indicate that secondary amide linkages are formed by the reaction between the genipin ester group and the chitosan amino group, yielding a polymeric network structure. A cytotoxic test revealed that 20 ppm in the culture medium is the threshold concentration of genipin over which it is cytotoxic to L929 fibroblasts. However, the chitosan and *bletilla striata* herbal extract not only were non-toxic but also promoted the viability and growth of L929 fibroblasts. These results verify that during the degradation of the GCB film, the degradable components of the GCB film not only have no cytotoxic effect on the *in vitro* proliferation of L929 fibroblasts but also promote cells, viability and growth. Additionally, the GCB film effectively supported cell attachment and growth. Finally, animal studies revealed that the bi-layered GCB/NWF-dressed wound was epithelialized, indicating perfect regeneration of damaged tissue, which allowed the GCB/NWF composite to be easily stripped from the wound surface without damaging the newly regenerated tissue. The results of experimental wound healing studies of laboratory rats demonstrated the effectiveness of the prepared GCB/NWF composite as a wound dressing material.

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