Preparation and evaluation of a novel wound dressing sheet comprised of β-glucan–chitosan complex

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1. Introduction

Traditional wound dressings, including absorbent cotton and gauzes, have been used to absorb wound exudates and thus maintain wound dryness and to prevent the invasion of bacteria into the wound. However, wound healing occurs more rapidly and successfully when epithelial cells can move unimpeded – in other words, when wounds are moist, receive adequate oxygen circulation to facilitate regeneration of cells and tissues, and are protected from bacterial invasions [1,2]. In recent years, many types of wound dressings and devices have targeted different aspects of the wound healing process. These dressings can be classified according to their function (e.g., absorbing moisture, facilitating debridement, functioning as an antibacterial, acting as an occlusive, adhering to the wound, enhancing healing, or mitigating pain), the materials from which they are constructed, including synthetic polymers (e.g., polyurethane, polyethylene, polyvinylpyrrolidone), and biomaterials (e.g., collagen, alginates, chitin, chitosan, or hyaluronic acid), and the physical form of the dressing (e.g., hydrocolloid, ointment, fiber, film, foam, or gel) [3]. It is important to have variety in dressing functions, materials, and forms, as they are needed for use on many different types of wounds (e.g., acute or chronic, different depths and areas, and different parts of body), for patients in a variety of health states, and for wounds at different stages of the healing process.

One material that has shown promise as a component of dressings is β-glucan, a polysaccharide comprised of β-linked D-glucose molecules. A variety of β-glucans have been isolated from various sources, such as fungi, baker’s yeast, barley, and seaweed. The physicochemical properties of β-glucans differ depending on characteristics of their primary structure, including linkage type, degree of branching, molecular weight, and conformation (e.g., triple helix, single helix, and random coil structures) [4]. Recent reports have shown that β-glucan plays a significant role in treating cancer, lowering blood cholesterol levels, reducing acute inflammatory responses, and acting as a biological response marker to enhance the immune system [5–8]. For instance, β-(1,3–1,6)-D-glucans isolated from black yeast (Aureobasidium pullulans) has attracted the attention of scientists because it enhances the immune system; in particular, it activates macrophages and accelerates the production of cytokines such as TNF-α, IL-6, and IL-12 [4,9].

Another material that shows promise as a component of dressings is chitosan (CS), a plentiful natural polysaccharide isolated mainly from crab shells. CS is comprised of linearly (1–4)-linked N-acetyl glucosamine and glucosamine residues. The physicochemical and biological properties of CS are influenced by its molecular weight and degree of deacetylation. Additionally, CS is non-toxic, biocompatible, and biodegradable [10,11]. Numerous studies have demonstrated that CS is an effective and safe vehicle for drug delivery.
for drug delivery [12–16] and can be used for implantable biomedical applications [17]. Furthermore, CS has also been reported to act as an antioxidant [18,19], to have analgesic effects on inflammatory pain [20], and to accelerate wound healing by activating and infiltrating polymorphonuclear cells and basic fibroblast growth factors in wounds [21,22]. In vitro studies have shown that CS can accelerate the proliferation of keratinocytes [23].

Traditional dressings have been made of animal materials, such as pigskin, which are biocompatible but induce immune reactions in humans and may be rejected. Also, dressings have been comprised of various synthetic polymers, which may become incorporated into granulation tissue, thereby remaining in the body once the wound is healed. As these examples indicate, dressings must contain a fine balance of characteristics; they should facilitate the quick and successful healing of wounds, be safe and biocompatible, and, if possible, contain a curative to enhance the healing process.

To meet this need, we have created a novel wound dressing from a complex of β-glucan and CS. This dressing is biocompatible, bioabsorbable, biodegradable, and has therapeutic efficacy. Here, we present the methods by which this dressing was fabricated, and we describe the results of an investigation of the dressing’s physiological properties and therapeutic efficacy when applied to all skin layer defects on a murine test subject.

2. Materials and methods

2.1. Materials

β-(1,3–1,6)-D-Glucan isolated from black yeast (Aureobasidium pullulans) was donated by ADEKA Co. (Tokyo, Japan). CS was donated by Ajinomoto Co. Inc. (Tokyo, Japan), Koyo Chemical Co. Ltd. (Osaka, Japan), Kyowa Technos Co. Ltd. (Tiba, Japan), Nipponkayaku Food Techno Inc. (Gunma, Japan), Yaegaki Bio-industry, Inc. (Hyogo, Japan), and Yaizu Suisankagaku Industry Co. Ltd. (Shizuoka, Japan), or purchased from Katokichi Co. Ltd. (Kagawa, Japan). Beschitin®W, a wound dressing material made from CS, was purchased from Unitika Ltd. (Aichi, Japan). All other chemicals were of reagent grade.

2.2. Preparation of sheets comprised of β-glucan–CS complex

The procedure for preparing sheets of β-glucan–CS complex is shown in Fig. 1. CS powder (0.1–2% w/w) was suspended in a solution of β-glucan (0.1–1% w/w) dissolved in ion-exchanged water. The suspension (2 g) was poured into a laboratory dish (inside diameter: 28 mm) and then laminated with ethanol (2 ml), which caused the solution to gel. After 2 h, the ethanol was removed by decantation and replaced with 2 ml of 0.1 M acetic acid buffer solution (pH 4.5) for 24 h. The permeation of the acetic acid buffer solution into the β-glucan solution caused the suspended CS to dissolve and form a complex with β-glucan. After 24 h, the supernatant was removed by decantation, allowing retrieval of the hydrogel sheet consisting of the β-glucan–CS complex. This sheet was dried at room temperature for >24 h to form the β-glucan–CS complex sheet.

The β-glucan sheet was prepared using a similar procedure. β-Glucan (0.1–2.0% w/w) without CS was dissolved in ion-exchanged water. The solution (2 g) was poured into a laboratory dish (inside diameter: 28 mm) and laminated with ethanol (2 ml), which caused the solution to gel. After 24 h, the ethanol was removed by decantation from the laboratory dish, and the hydrogel sheet was retrieved and dried at room temperature for >24 h.

2.3. Measuring X-ray diffraction of the β-glucan–CS complex, β-glucan, and CS

X-ray diffraction was carried out using a diffractometer (D8 DISCOVER with GADDS, Bruker AXS K.K., Kanagawa, Japan) and was operated using Cu Kα radiation at 45 kV and 360 mA. The scanning rate was 2°/min over a 2θ range of 3–100°.

2.4. Rheological study

The rheological properties of the β-glucan–CS complex sheet were determined using a rheometer (SUN RHEO TEX SD-700®, Sun Scientific Co. Ltd., Tokyo, Japan). To do this procedure, the dried sheet was fixed with polyurethane film (Tegaderm™ transparent dressing, 3 M, Maplewood, MN, USA) and was observed using Cu Kα radiation at 45 kV and 360 mA. The scanning rate was 2°/min over a 2θ range of 3–100°.

2.5. Measuring the biodegradability of the β-glucan–CS complex sheet, β-glucan sheet, and Beschitin®W sheet

Air (3 ml) was injected subcutaneously into the dorsal surfaces of 6-week-old male ddY mice to form air pouches (AP). Oval APs were formed after an additional 1 ml of air was injected at days 1 and 4. On the seventh day after air was first injected, mice were anesthetized with ether and implanted with sheets via 1-cm incisions in the APs. After a predetermined period of time (3 days, 1 week, and 2 weeks after implantation), mice were euthanized via cervical dislocation. The sheets were retrieved, and their biodegradation was assessed.

2.6. Evaluating therapeutic efficacy of β-glucan–CS complex sheets in each mouse

Six-week-old male ddY mice were placed under ether anesthesia, their limbs were restrained, and their dorsal surface hair was removed with hair clippers. The dorsal surface of each mouse was disinfected with ethanol, after which a circular patch of skin, approximately 1 cm in diameter and penetrating all skin layers, was excised from each mouse. The major and minor axes of the wounds were measured by slide calipers. β-Glucan–CS complex sheets were immersed into physiological saline in order to wash out salts, then immediately placed on the wound (one per mouse). Sheets were fixed with polyurethane film (Tegaderm™ transparent dressing, 3 M, Maplewood, MN, USA).

As comparisons, a β-glucan sheet and a Beschitin®W sheet (Unitika Ltd., Aichi, Japan) were applied using the same techniques. Furthermore, no-dressing application treatments (where the
wound was covered only with Tegaderm™ polyurethane film) and no-application treatments (a wound without dressing or film) were also prepared as controls. After 3, 5, 7, 10, 12, and 14 days from the application of the sheet, mice were restrained, and all films and dressings were removed. For each treatment, the major and minor axes of the wounds were measured. The rate of change of the wound area was calculated by the following equation and used as an estimate of the curative effect of the treatment:

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\% \text{ of wound remaining} = \frac{\text{wound area after treatment}}{\text{wound area prior to treatment}} \times 100
\]

All research protocols were approved by the Committee for Animal Research at Hokuriku University.

2.7. Statistical analyses

Data are represented as mean ± SD and were analyzed with F-tests and Student’s t-tests.

3. Results and discussion

3.1. Properties of the β-glucan–CS complex sheet

Dissolution of the CS suspended in the β-glucan solution was aided by immersion in an acetic acid buffer solution, which triggered salt formation between some amino groups of CS and acetic acid. These salts dissociated after contacting β-glucan, leading to formation of a hydrogel sheet comprised of β-glucan–CS complex. The final product, a colorless and transparent sheet, was obtained by drying the hydrogel at room temperature. If β-glucan solution and CS acetic acid solution were mixed, then fibrous gels were spontaneously formed preventing such a sheet from forming. Unlike β-glucan powder, which dissolves in both acidic and basic aqueous solutions, and CS powder, which dissolves in weak acidic aqueous solutions, the complex sheet did not dissolve in either acidic or basic aqueous solutions. The difference in properties between the original components (β-glucan and CS) and the new sheet indicated that the β-glucan–CS complex had successfully been formed. Furthermore, the X-ray diffraction pattern from sheets comprised of β-glucan–CS complex differed from either that of β-glucan or CS (Fig. 2). The difference in diffraction patterns also suggested the formation of complex had occurred.

High concentrations (e.g., those >2.0%) of β-glucan solution resulted in high viscosity solutions, making them difficult to spread into a laboratory dish. However, very low concentrations (e.g., those <0.1%) did not lead to sheet formation. Successful sheet formation only occurred when moderate concentrations (e.g., approximately 0.1–1%) of β-glucan solution were mixed with 0.5% or more of CS. It was also possible to form the transparent and colorless sheet without the addition of CS, which was done by simply evaporating the aqueous component of a viscous solution of >0.5% β-glucan.

In general, sheets prepared with lower concentrations of β-glucan broke at much lower stress levels than sheets prepared with higher concentrations, up to a maximum of 1.0% β-glucan (Fig. 3). This maximum limit was likely due to the fact that higher β-glucan concentrations increased the thickness of the sheets and therefore reduced their pliability. Sheets composed of only β-glucan dissolved easily when placed in contact with water.

The rheological properties of the β-glucan–CS complex sheets varied according to the concentrations of β-glucan and CS used to form the sheets. At the lowest concentrations of β-glucan, sheets broke at increasingly higher stress levels as the concentration of CS was increased (Fig. 4a and b). However, at the highest β-glucan concentration (1.0%), the stress was maximized when there was no or only 1.0% CS (Fig. 4c). Thus, the optimum positive CS concentration appears to be 1%, which produces a sheet where stress = 920 ± 90 kPa, and strain = 3.3 ± 0.2 mm (Table 1). These values are superior to those of Beschitin™W (stress = 490 ± 40 kPa; strain = 2.0 ± 0.2 mm) and comparable to those of the food-packing film, Saran Wrap® (Asahikasei Co. Ltd., Tokyo, Japan) (stress = 980 ± 10 kPa; strain = 5.8 ± 0.2 mm). This result indicates that the β-glucan–CS complex sheet is more than capable of handling the stress and strain to which wound dressings are often subjected.

Formation of the sheet may be influenced by various CS properties, such as molecular weight and degree of deacetylation. However, the strength of the sheet did not appear to be highly correlated with these properties in the 16 CS species examined here, which ranged in size from 31,000 to 2,826,000 Da and were 53–100% deacetylated (Table 1). This lack of correlation was likely due to differences in inter- or intra-molecular attractive and repulsive forces in the CS polymers. These forces are associated with differences in molecular weight, degree of deacetylation, and distribution of acetamide groups of the CS.

3.2. Biodegradability of the sheet

Biodegradability of CS is governed by various properties, particularly the degree of deacetylation. Decrease in deacetylation leads to acceleration in biodegradation [12]. β-glucan degrades via phagocytosis by macrophages and neutrophils [24]. In a previous study, the biodegradation of processed dosage forms (e.g., gel
beads) composed of only CS or a CS-containing complex were found to be influenced by the CS properties [25]. Thus, we hypothesized that the biodegradability of the β-glucan–CS complex sheet would be influenced primarily by the properties of CS. Additionally, we hypothesized that wound healing activities would be accelerated after the release of β-glucan and CS during biodegradation, due to the physiological activities of these compounds.

Sheets comprised of β-glucan–CS complex were found to degrade more quickly when prepared with less-deacetylated CS (Table 1). For instance, sheets with ≤70%-deacetylated CS degraded within 3 days after implantation, whereas sheets with >80%-deacetylated CS maintained their shape for over a week. After two weeks, some sheets had dissolved or completely disappeared, whereas others became very soft but continued to maintain their shapes (Table 1). Regardless, more biodegradation occurred among the β-glucan–CS complex sheets than in Beschitin™W, which maintained its shape even after 2 weeks. However, the β-glucan only sheet degraded completely within 3 days after implantation.

As biodegradation proceeds, β-glucans and CS will gradually be released; the rate at which this occurs can be controlled by changing the properties of the CS used to create the sheets. It is ideal for a wound dressing to maintain its shape for the duration of the application period in order to create an optimum environment for rapid and successful wound healing – that is, to allow epithelial cells to move unimpeded, to retain moisture, to facilitate oxygen circulation needed for cell and tissue regeneration, and to prevent bacterial invasions. Therefore, it is important that CS properties are carefully selected when forming sheets to optimize their effectiveness. Furthermore, when wound dressing fragments are left at the wound site after the healing process, it would be ideal if they could be absorbed by the body via biodegradation or phagocytosis. Thus, because β-glucan–CS complex sheets are biocompatible, bioabsorbable, and biodegradable, they appear to be ideal candidates for wound dressings.

3.3. Therapeutic efficacy of β-glucan–CS complex sheets

Wounds that were dressed with β-glucan–CS complex sheets rapidly decreased in size during the first 7 days following treatment, and then they continued to decrease at a slower rate over the remaining week (Fig. 5). In addition, the β-glucan–CS complex sheets did not dissolve during the application period; did not adhere to the wound, and were easy to remove without ripping the skin. Finally, wounds healed under the β-glucan–CS complex sheet demonstrated better tissue quality with less scarring.

Wounds dressed with Beschitin™W + Tegaderm™ showed healing trajectories similar to those of the complex sheets, but wounds persisted at a larger size for longer timescales compared with the β-glucan–CS complex sheets prepared with CS(E) species, which is the more rapidly biodegradable CS species. Wounds that received only Tegaderm™ and no-application treatments had the slowest rates of healing. Mice receiving the no-application treatment formed scabs on their wounds in response to drying, but we rarely observed re-epithelialization of the skin wound via formation of normal skin tissue under the scabs. The no-dressing treatment (application of Tegaderm™ only) maintained a moist wound environment throughout the experiment; however, excess wound fluid was not observed. Both re-epithelialization and granulation tissue was also rarely observed. Wounds dressed with only a β-glucan sheet showed healing trajectories similar to those of the no-dressing treatment group. The β-glucan sheet immediately dissolved on the wound indicating that the therapeutic effects of this sheet were negligible.

In general, injury to the skin initiates a cascade of events, including inflammation, new tissue formation, and tissue remodeling, which finally lead to reconstruction of the wounded area. The repair process is initiated immediately after injury by the release of various growth factors, cytokines, and low-molecular-weight compounds from the serum of injured blood vessels and de-granulating platelets. Inflammatory cells such as neutrophils, lymphocytes, and macrophages invade the wound tissue within a few hours after injury, and they are an important source of growth factors and cytokines, which initiate the proliferation phase of wound repair. The next stage of wound repair occurs with the migration and proliferation of keratinocytes at the wound edge and is followed by proliferation of dermal fibroblasts in the neighborhood of the wound. During the last stage, the wound is completely filled with

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**Fig. 4.** Effect of CS concentration on the rheological properties of β-glucan–CS complex sheets at various concentrations of β-glucan: (a) 0.1% β-glucan; (b) 0.5% β-glucan; (c) 1.0% β-glucan. CS (molecular weight = 84.2 × 10⁴ Da; degree of deacetylation: 95%) concentration: ○, 0%; ▲, 0.1%; ▲, 0.5%; ▲, 1.0%; △, 2.0%. Data represented by the mean ± SD (n = 3).
granulation tissue and completely covered with a neo-epidermis [26].

This process can be aided and hastened by the application of films and/or dressings. Wounds treated with β-glucan–CS complex sheets exuded more wound fluid than those that received the Beschitin®W/C210W treatment (Fig. 6 and 3 days). These fluids contain various growth factors, cytokines, and low-molecular-weight compounds which are needed for wound repair. β-glucan, particularly the variety used in this study (β-(1,3-1,6)-D-glucan isolated from black yeast), has been reported to enhance the immune system by activating inflammatory cells and accelerating cytokine production [4,9]. CS has also been demonstrated to accelerate wound healing by activating and infiltrating polymorphonuclear cells, by up-regulating basic fibroblast growth factors, and by encouraging proliferation of keratinocytes at wound sites [21–23]. Thus, it appears that sheets comprised of rapidly-biodegrading CS species promote wound recovery by releasing greater amounts of β-glucan and CS molecules. These molecules lead to increased activity of inflammatory cells and activation of factors that regulate wound repair (e.g., growth factors and cytokines). Thus, the β-glucan–CS complex sheet accelerates the re-epithelialization of the skin wound.

### 4. Conclusion

A transparent, biocompatible, bioabsorbable, and biodegradable sheet was manufactured by forming a complex of β-glucan with CS.

### Table 1
Rheological properties and biodegradability of β-glucan–CS complex sheets prepared with different species of CS.

<table>
<thead>
<tr>
<th>CS species</th>
<th>MW (×10^2 Da)</th>
<th>DA (%)</th>
<th>Rheological properties</th>
<th>Biodegradability</th>
</tr>
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<tbody>
<tr>
<td>Stres (kPa)</td>
<td>Strain (mm)</td>
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<td></td>
<td></td>
<td></td>
<td>3 days</td>
<td>1 week</td>
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<tr>
<td>A</td>
<td>3.1</td>
<td>87</td>
<td>390 ± 170</td>
<td>±</td>
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<tr>
<td>B</td>
<td>4.4</td>
<td>96</td>
<td>410 ± 230</td>
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</tr>
<tr>
<td>C</td>
<td>5.7</td>
<td>99</td>
<td>550 ± 70</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>6.6</td>
<td>97</td>
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<td>E</td>
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<tr>
<td>F</td>
<td>8.3</td>
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<td>G</td>
<td>17.9</td>
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<td>H</td>
<td>44.8</td>
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<tr>
<td>I</td>
<td>52.5</td>
<td>85</td>
<td>660 ± 260</td>
<td>+</td>
</tr>
<tr>
<td>J</td>
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<td>93</td>
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<td>L</td>
<td>84.2</td>
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<td>S</td>
<td>490.0</td>
<td>52</td>
<td>680 ± 90</td>
<td>±</td>
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</table>

MW: molecular weight; DA: degree of deacetylation. Data of rheological properties are represented by the mean ± SD (n = 3). Shape of β-glucan–CS complex sheets after implantation: +, complete maintenance of shape; ±, dissolution or failure to maintain shape; –, complete disappearance. β-glucan–CS complex sheets were prepared with 1% of β-glucan and 1% of CS.
Biodegradation of the sheet leads to the release of β-glucan and CS, which accelerates wound repair by activating macrophages and cytokines. By manipulating the properties of the CS used to create the sheet, we found that we were able to control the biodegradation of the sheet, thereby adjusting the release rate of β-glucan and CS. The complex sheet did not dissolve during the application period, did not adhere to the wound, and was easy to remove. Additionally, its components are naturally biodegradable and bioabsorbable. One further attraction of this design is that the sheet’s curative effects resulted from the materials of which it is comprised, rather than any additives. Cumulatively, these results indicate that the β-glucan–CS complex sheet is a promising new wound dressing.

References