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Photocrosslinkable gellan gum film as an anti-adhesion barrier

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ABSTRACT

The purpose of this study was to develop a gellan gum-based film which could be photocrosslinked for medical applications. Gellan gum was grafted with cinnamate to yield the photo crosslinkable polymer (gellan gum-cin). This material had 14.7% of its D-galacturonic residues reacted with cinnamate groups and displayed maximum absorption at 254 nm. Investigation of the photochemical properties showed that the crosslinking efficiency was 82% after 16 min of UV irradiation. The anti-adhesion films prepared from gellan gum-cin polymers exhibited high gel contents (88 ± 2%) and suitable mechanical properties. When implanted into rats, the gellan gum-cin film exhibited the most promising anti-adhesion potential in 2 out of 10 rats without forming any tissue adhesion. Furthermore, the gellan gum-cin film could effectively inhibit inflammation in rats based on the results of fluid leukocyte analyses. The gellan gum-cin film thus has potential in clinical applications.

1. Introduction

Gellan gum is a linear, anionic extracellular polysaccharide from *Pseudomonas elodea* with repeating tetrasaccharide units of D-glucose, D-glucuronic acid, D-glucose, and L-rhamnose (Jansson, Lindberg, & Sandford, 1983; O'Neil, Selvendran, & Morris, 1983). Gellan gum is a food additive that functions as a stabilizer, thickening agent, and structuring and versatile gelling agent in a wide variety of foods. Recently, gellan gum has been investigated as a candidate material for biomedical engineering because of its biocompatibility and low cytotoxicity (Oliveira et al., 2010; Silva-Correia et al., 2011). Gellan gum has also been tested as a drug-delivery carrier, cell carrier, guided bone-regeneration material, and wound dressing (Chang et al., 2010; Lee, Chen, & Tsao, 2010). The stable cross-linked structure of gellan gum can be obtained in the presence of metallic cations or by forming bonds between gellan gum molecular chains and chemical cross-linkers, such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Although the polysaccharide can be cross-linked with Ca^{2+} ions, the mechanical properties of polysaccharide are fragile and less malleable (Ichibouji, Miyazaki, Ishida, Sugino, & Ohtsuki, 2009). When implanted, tissue calcification occurs, which limits the biomedical application of Ca^{2+}-cross-linked gellan gum. In addition, chemical cross-linkers can be cytotoxic due to dosage responses and cross-linker residues (Powell & Boyce, 2006).

To develop a non-toxic method of cross-linking gellan gum that can be applied in biomedicine is the main purpose of this research work. Crosslinking via the photodimerization of polymeric systems has been utilized in various applications. In this study, we designed a new photocrosslinkable gellan gum molecule that contains a cinnamate moiety and may be used for medicinal purposes. The crosslink mechanism is based on the π-electron density of the photoactive chromophore, with dimerization of the cinnamate groups occurring presumably as a result of [2 + 2]π-electron cycloaddition (Dong et al., 2005). The reaction does not require the addition of a light-sensitive initiator. Cinnamate is a natural tropane alkaloid found within the Erythroxylum coca plant that possesses anti-inflammatory and non-toxic properties (Ballabeni et al., 2010). In this study, cinnamate functions not only as a cross-linking agent but also as an anti-inflammatory drug.

Various types of films made of polysaccharides have been reported to reduce adhesion formation, including Dextran-70, Interceed, and Seprafilm™ (Robertson et al., 2010), but have not fully satisfied the requirements for clinical implementation. An ideal adhesion-prevention product should be resolvable, non-reactive, easy to apply, and capable of being fixed. In a previous study, we demonstrated that gellan gum could prevent fibroblast adhesion and migration. In this report, we describe the evaluation of the efficacy of a photosensitive gellan gum film (denoted as GG-Cin film) in reducing postoperative adhesion formation in a rat model.

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2. Methods

2.1. Dissolution of gellan gum in dimethyl sulfoxide (DMSO)

To render gellan gum soluble in DMSO, the sodium ions of gellan gum were exchanged with the lipophilic tetrabutylammonium (TBA) ion (Oudshoorn, Rissmann, Bouwstra, & Hennink, 2007). Ion exchange was performed using Dowex® 50W-X8 cation-exchange resin (1.8 mmol/g exchange capacity; Fluka 44519). The Dowex® resin was incubated with a large excess of TBA (1:2.77 molar ratio of the exchange capacity of Dowex® to TBA) dissolved in 50 ml deionized water for 1 h and washed extensively with water. Next, the resin was transferred into 1% (w/w) gellan gum solution in water (1:10 molar ratio of the carboxyl groups of gellan gum to Dowex®-TBA) and mixed for 2 h at room temperature. The mixture was then centrifuged for 10 min at 3000 rpm to remove the resin. The obtained gellan gum-TBA solution was lyophilized and used for chemical modification with a photosensitive group.

2.2. Synthesis of gellan gum-cinnamate in DMSO

Gellan gum-TBA was dissolved in DMSO (1%, w/w). Cinnamyl bromide was dissolved in DMSO to a concentration of 4% (w/w). A mixture of gellan gum-TBA solution and cinnamyl bromide solution was stirred at 50 °C for 48 h. The mole ratio of cinnamate to gellan gum carboxyl residues was 5:1. The gellan gum-cinnamate (GG-Cin) product was purified by ethanol precipitation. The purified gellan gum-cin was analyzed by ^{1}H NMR (500 MHz, Bruker Advance DRX500).

2.3. Photochemical properties of gellan gum-cin

The photoreactivity of gellan gum-cin was studied by dissolving it in DMSO to a concentration of 0.1% (w/v) and exposing to UV light at 254 nm using a mercury lamp (Cole-Parmer 9815-series lamps 100 W) for different intervals of time. After each irradiation period (2 min), UV spectra were recorded on a scanning spectrophotometer (Milton Roy Spectronic 3000 array). The crosslinking efficiency was determined by calculating the percent conversion of photoactive chromophores using the following equation (Dong et al., 2005):

$$\%\ \text{crosslinking} = \frac{A_t - A_0}{A_\alpha - A_0} \times 100$$

where A_0, A_t, and A_α are respectively the absorbance values at time 0, time t, and time α after which no further changes were observed in the absorbance.

2.4. Preparation of gellan gum-cin film

Gellan gum-cin (0.2 g) was dissolved in 1.5 ml DMSO and then mixed with 13.5 ml deionized water. The solution was poured onto a glass dish (diameter 5 cm) and evaporated at 50 °C until the weight of the film was constant. To prevent dissolution of the non-cross-linked gellan gum-cin film in the aqueous solution, the film was immersed in ethanol and irradiated with UV light (Cole-Parmer 9815-series Lamps 100 W) for 30 min. The cross-linked gellan gum-cin film was washed with 95% ethanol three times and then dried at room temperature.

2.5. Characterizations of the cross-linked gellan gum-cin film

An electrical thickness tester (mitutoyo, MDC-25 SB) was used to measure the thickness of the gellan gum-cin film. We used the FTIR-L396A (Perkin-Elmer) to analyze the properties of the chemical functional groups of the cross-linked gellan gum-cin film. The analysis of the gel content of the cross-linked gellan gum-cin film was performed as follows. After drying, we weighed the cross-linked film (W_1) and then swelled it in DDW at 37 °C for 24 h. After removing the wet film from the solution, the film was dried in a vacuum oven for 12 h at 60 °C and then weighed again (W_2). The gel content (%) was 100 (W_2/W_1).

The gellan gum-cin film was cut into 1 cm × 5 cm pieces (Mathew & Abraham, 2008). We then used the H1-KS testing machine (Tinius Olsen) with a crosshead speed of 5 mm/min to measure the mechanical properties of the gellan gum-cin films and to automatically record the mechanical parameters.

2.6. Animal implant study

Twenty Sprague-Dawley rats (200–250 g) were tested in a surgical research laboratory. Aseptic midline laparotomies were conducted while the animals were anesthetized with 4% trichloroacetaldehyde monohydrate (1 ml/100 g). The distal 3 cm of the cecum and opposing abdominal wall were scraped with a scalpel until the serosal surface was disrupted and hemorrhaged but not perforated. The denuded peritoneal wall was then covered with a gellan gum-cin film (diameter: 1.0 cm). The rats in the control group were not covered with any anti-adhesion film. Contact between the cecum and opposing peritoneal wall was maintained in all animal groups with two nonoccluding loops of 4/0 polypropylene sutures placed 2 cm apart. After completion of the procedure, the abdomen was closed in a double layer using 4/0 polypropylene in a continuous fashion. The experimental rats were sacrificed on day 3 or 7 after surgery to examine the process of adhesion formation at the injured site (Peng et al., 2011). Adhesions were scored in a blinded manner according to the method of Zuhlke HV et al. (Table 1), where grade 0 indicates no adhesions and grade 4 indicates firm extensive adhesions that are dissectable only with sharp instruments and almost unavoidable organ damage. The abdominal wall of the injured site was removed and fixed in 10% formalin solution. The tissues were processed by the standard procedure for histological examinations, and their thin sections were examined after staining with hematoxylin–eosin (H–E).

2.7. Peritoneal fluid analysis

Peritoneal fluids were collected before the operated animals were sacrificed on day 3 or 7 after surgery. The peritoneal fluid was aspirated through a pipette with a bulb tip after 3 ml of the DMEM containing heparin was injected into the peritoneal cavity. Turk's solution (0.01% Giemsa stain and 3% acetic acid) was used to stain white blood cells, and the number of neutrophils in the collected fluid was determined by cell counting using a hemocytometer.

2.8. Statistical analysis

Each of the experiments was repeated at least five times, and the values were expressed as the means ± standard deviations. For comparison between two groups of data, Student's t-test was performed. Differences were considered to be statistically significant at $P < 0.05$.

Table 1
Grading of adhesion according to Zuhlke (grade description).

0	No adhesions
1	Filmy, fibrin adhesions, easily removed by blunt dissection (mild)
2	Fibrous adhesions, easily dissected (moderate)
3	Thick fibrous adhesions, dissectable (severe)
4	Thick fibrous adhesions, not dissectable without damage to the adherent tissue (very severe)

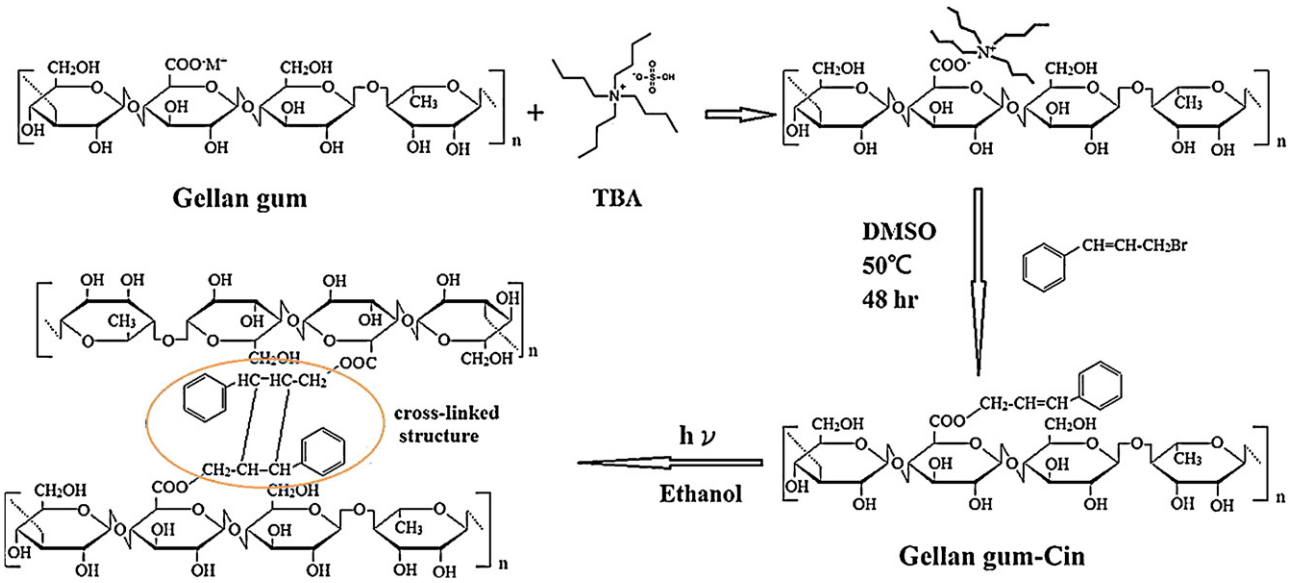

Fig. 1. The photocrosslinking mechanism of gellan gum.

3. Results and discussion

3.1. NMR characterization of gellan gum-cin

We have developed a method to crosslink gellan gum using pendant photofunctional groups, such as cinnamate. Gellan gum was grafted with cinnamate to yield gellan gum-cin in DMSO. Gellan gum-cin polymers having α,β-unsaturated carbonyl groups in the backbone underwent crosslinking upon irradiation with UV light. The reaction mechanism is depicted in Fig. 1. The gellan gum-cin polymer was analyzed based on the ^{1}H NMR spectra results (Fig. 2). These results showed the presence of characteristic peaks that correspond to —CH of rhamnose (δ 5.2–5.6 ppm), —CH of glucuronic acid (δ 4.9–5.1 ppm), —CH of glucose (δ 4.0–4.8 ppm) and —CH_3 of rhamnose (δ 1.2 ppm) (Daniela F Coutinho et al., 2010). The spectrum confirms the incorporation of the cinnamate group by the presence of methylidyne proton peaks at 6.3 and 7.5 ppm and a phenyl proton peak at 7.2–7.5 ppm. The degree of cinnamate substitution can be conveniently determined by comparing the integrated intensity of the phenyl and —CH=CH— peaks of the cinnamate group to the integral of the —CH_3 protons (δ 1.2 ppm) of gellan gum. Accordingly, the degree of cinnamate substitution was approximately 14.7% (Dong et al., 2005).

3.2. FTIR characterization of gellan gum-cin

Fig. 3a and b shows the FTIR spectrograms of gellan gum and gellan gum-cin polymers. Fig. 3a shows the assignment of the absorption band at 3309 cm^{-1} to the stretching of the —OH groups in gellan gum (Sudhamani, Prasad, & Udaya Sankar, 2003). The band at 2917 cm^{-1} is due to the stretching vibrations of the —CH_2 group, whereas the bands appearing at 1148 and 1015 cm^{-1} are due to ethereal and hydroxylic C—O stretching. The peaks at 1603 and 1403 cm^{-1} can be assigned to the characteristic absorption band

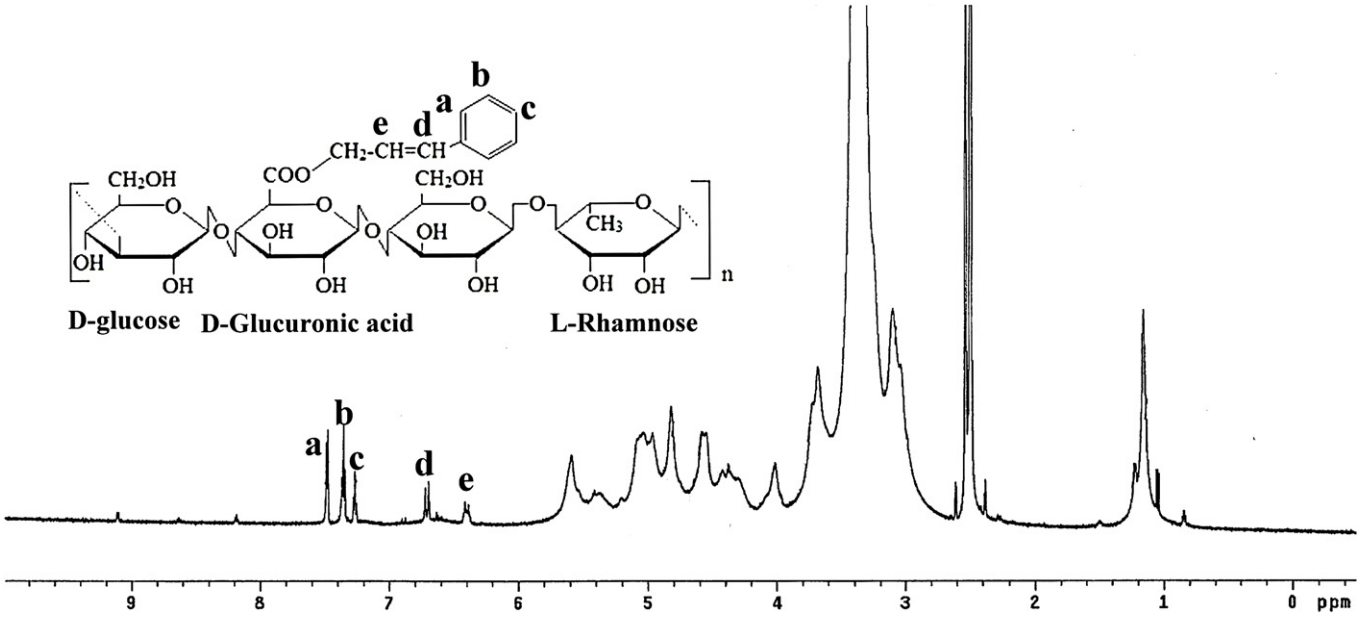

Fig. 2. NMR spectrum of gellan gum-cin.

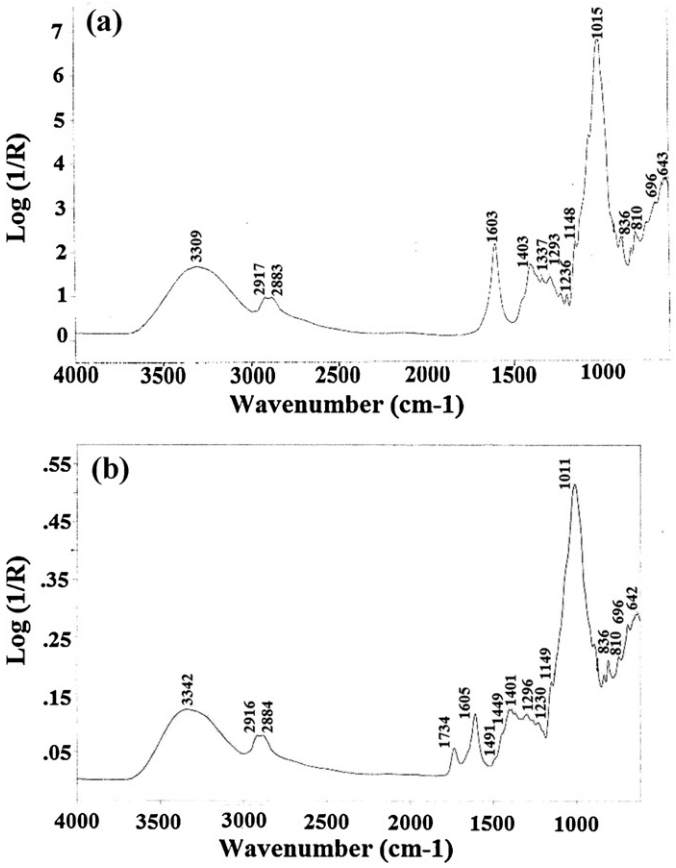

Fig. 3. FTIR spectra of gellan gum (a) and gellan gum-cin (b).

of carboxyl in gellan gum. The bending vibration of C—H appears at 835 cm^{-1}. Fig. 3b shows the FTIR spectrogram of gellan gum-cin; the most prominent difference in the spectrum between gellan gum and gellan gum-cin appeared as a new absorption peak at 1734 cm^{-1}, which was assigned to the C=O ester group. The C=C stretching vibration of the benzene ring in the cinnamate groups appears at 1449 and 1491 cm^{-1} (Francisco, Maria Jose, & Pedro Antonio, 2007; Sung et al., 2004). It also caused the absorption peak of the —OH groups to shift to a higher wave number, 3342 cm^{-1}, and that of the C—O stretching to shift from 1015 to 1011 cm^{-1}. These data indicate that the new absorption peak is not caused by residual cinnamate and confirms that the covalent grafting reaction was successful.

3.3. Photoreactivity measurements

The UV spectrum of gellan gum-cin polymer is shown in Fig. 4. Gellan gum-cin exhibited an absorption around 254 nm due to the π–π^* transition of the double band present in the polymer chain. When the gellan gum-cin polymer were subjected to the UV irradiation, there is a change in the UV absorption maximum at 254 nm due to the formation of cyclobutane ring. This cyclobutane ring formation is responsible for the decrease in the absorption upon irradiation with UV light. This type of decrease maximum upon irradiation was reported earlier (Watanabe, Ichimura, & Suda, 1986). The photosensitive gellan gum polymers with pendant chalcone groups (α,β-unsaturated carbonyl) that undergo a (2+2) photocycloaddition reaction upon UV irradiation are regarded as negative-type photoresists (Allen, Mallon, Timms, Green, & Catalina, 1993; Nagata & Inaki, 2009). The crosslinking efficiency of the gellan gum-cin increased with the duration of light irradiation, as shown in Fig. 5. Within 4 min, the crosslinking efficiency was

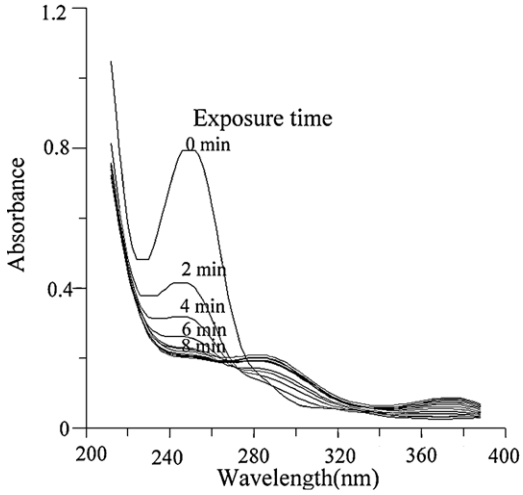

Fig. 4. The change of UV spectra of the photosensitive gellan gum-cin induced by irradiation of UV light through the filter.

82% and was nearly complete within 16 min. These results suggest that a short exposure time was enough to induce the cross-linking reaction of the photopolymer.

3.4. Physical properties of crosslinked gellan gum-cin film

The gellan gum-cin film was crosslinked via short wavelength UV irradiation (254 nm). The average thickness of the films was 24 ± 2 μm. The prepared film was soft, flexible, transparent, and capable of being fixed in position. The gel content of a film is related to the crosslinking density of the film (Nagasawa, Yagi, Kume, & Yoshii, 2004). The crosslinked gellan gum-cin film had a high gel content of approximately 88 ± 2%. However, the non-cross-linked gellan gum-cin film was rapidly swollen with water. For clinical use, the most important mechanical properties of anti-adhesion film are tensile strength and elongation. Mechanical testing revealed that the maximum tensile strength and elongation at break of the non-UV-irradiated film were 31.4 ± 4.8 Mpa and 8.2%, respectively. The maximum tensile strength and elongation at break of the UV crosslinked film were 42.6 ± 6.1 Mpa and 6.8%. It was observed that tensile strength of the crosslinked film was slightly increased and the film became stiffer. Vijayabaskar, Tikku, Bhowmick, and Anil

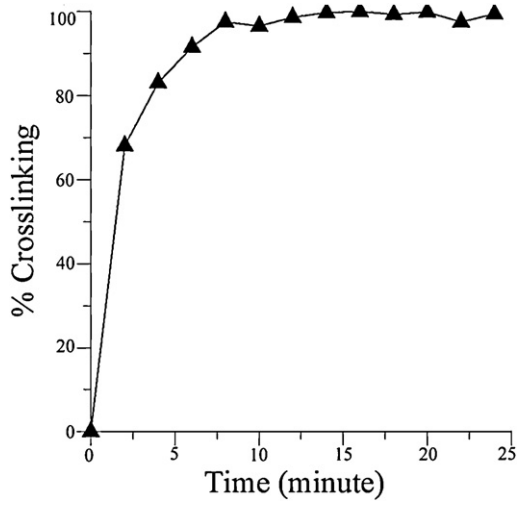

Fig. 5. The effect of irradiation time on the extent of crosslinking of the gellam gum-cin.

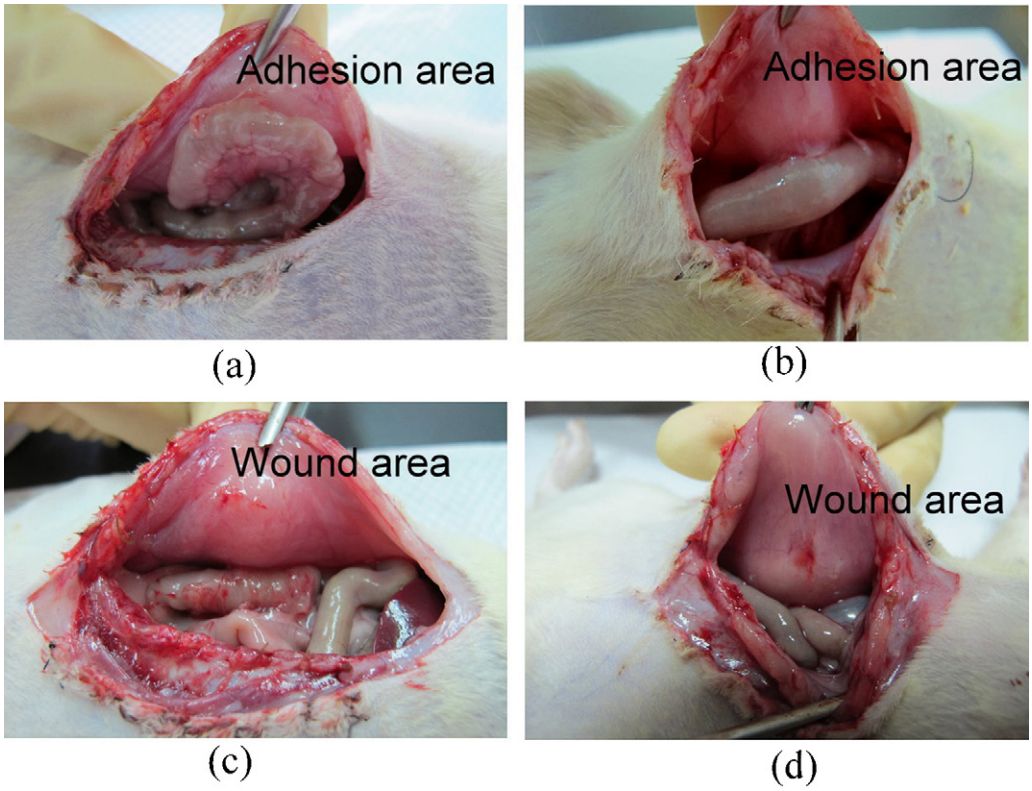

Fig. 6. Repair of the injured sites between the cecum and the peritoneal wall in the operated rats 3 and 7 days after surgery: (a and b) adhesion formation (control group) and (c and d) treated with gellan gum-cin film without adhesion formation.

(2006) indicated that the tensile strength of a polymer is closely correlated to the density of cross-linking. At high cross-link density, the segments of macromolecules become immobile, which causes the system to become stiff and show a decrease in elasticity. At present, no standards regarding the mechanical properties of anti-adhesion films are available for clinical evaluation. In this study, the vivo evaluation of gellan gum-cin films for the prevention of post-operative adhesion showed that the films had sufficient mechanical strength and easy to apply.

3.5. *In vivo evaluation of gellan gum-cin film for the prevention of postoperative adhesion*

The occurrence of tissue adhesion between the cecum and the peritoneum was examined on the 3rd and 7th days after surgery. In the control group (without membrane), adhesion of the cecum to the peritoneal wall was found in 5 of the total 5 rats operated (adhesion incidences 100%) on day 3 and day 7 after surgery (Fig. 6a and b). The adhesion scores of the control group were between 3 (severe) and 4 (very severe). In contrast, the gellan gun-cin film effectively prevented tissue adhesion in all rats operated (5 rats operated) on day 3 after surgery and reduced adhesion incidences by 60% (3 out of 5 rats operated) on day 7 after surgery (Fig. 6c and d). The adhesion scores of the experimental group were between 0 (no adhesion) and 1 (mild). Superior anti-adhesion capabilities were demonstrated by the gellan gum-cin film throughout the observation period.

Tissues surrounding the injured sites were dissected and examined. Photomicrographs of the sectioned, H&E-stained tissues on the 3rd and 7th day of the repairing process are shown in Fig. 7. In the control group, newly formed dense adhesive tissue was found between the peritoneal wall and the mucosa of the cecum. The adhesion area was also covered by thick fibrous tissue and contained a thick layer of fibroblast. With the treated gellan gum-cin film, on the 3rd day after surgery, the surgical lesions had not completely healed and did not form adhesive tissue between the peritoneal wall and the mucosa of the cecum. On the 7th day after surgery, the tissues around the surgical lesions had almost completely healed. Histological observation showed that on the 3rd and 7th days following surgery inflammatory cells were found around the surgical lesions in all groups. The number of inflammatory cells was counted using a cell-counting method.

3.6. *Quantitative analysis of the inflammatory cells*

We also assessed the number of peritoneal fluid neutrophils to evaluate whether the gellan gum-cin film had an anti-inflammatory capacity. The results showed that on the 3rd and 7th days following surgery, the number of neutrophils in the control group was $2.0 \pm 0.6 \times 10^5$ and $1.7 \pm 0.5 \times 10^5$ cells/ml, respectively, whereas the number of neutrophils in the experimental group was $1.1 \pm 0.2 \times 10^5$ and $0.9 \pm 0.5 \times 10^5$ cells/ml, respectively. For all test groups, the number of neutrophils reached a maximum within the first 3 days after surgery and then gradually decreased over the 7-day period of observation. On the 3rd day after surgery, the number of neutrophils in the control group was 1.81 (P-value 0.046) times that of the experimental group. On the 7th day after surgery, the number of neutrophils in the control group was 1.88 (P-value 0.0008) times that of the experimental group. Neutrophils are the predominant inflammatory cell type found in a wound during the first 7 days after injury. After injury, the normal healing process leads to inflammation and some scarring, which patches the damaged tissue. However, if an injury is not properly addressed, inflammation and scar tissue (adhesion fibrosis) can become more severe, leading to the beginning of a chronic injury cycle (Delavary, van der Veer, van Egmond, Niessen, & Beelen, 2011). Our results

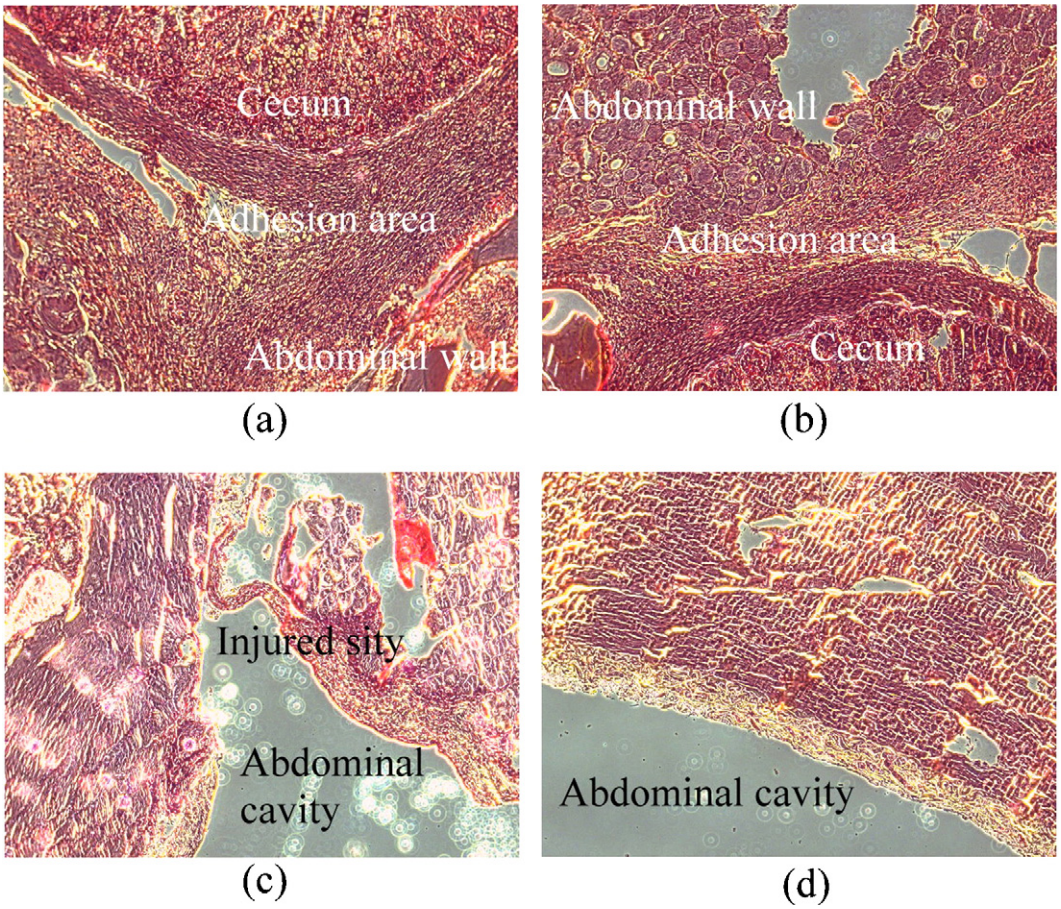

Fig. 7. Histological observation of the wound site in the operated rats 3 and 7 days after surgery: (a and b) control group and (c and d) treated with gellan gum-cin film. Thin sections were stained with H–E (100×).

demonstrated that a gellan gum-cin film could effectively inhibit inflammation in rats. In this study, gellan gum film plays a role not only as a physical barrier for the separation of wounded tissues after surgery but also as an anti-inflammatory drug carrier.

4. Conclusion

Various types of biodegradable films based on polysaccharide have been developed for anti-adhesion. Polysaccharides have high amounts of free carboxyl groups. At neutral pH, these polysaccharides and living cells are negatively charged. Stronger negative charges on the polysaccharide film surface provide long-range electrostatic repulsion, which can prevent the rapid adhesion of cells (Vitte, Benoliel, Pierres, & Bongrand, 2004). In addition, the details of cell–polysaccharide interaction remain largely uncharacterized. In this study, we have developed a new method to cross-link gellan gum using the cinnamate group as the cross-linker and designed a novel anti-adhesion film base on microbial polysaccharide gellan gum. The results of the in vitro characterization and in vivo evaluation of post-surgery anti-adhesion capability demonstrated that gellan gum-cin film has great potential for future use in clinical applications.

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