行政院國家科學委員會補助

大專學生參與專題研究計畫研究成果報告

＊ ********* ** ＊
＊ 計 畫 ＊
＊ ： 感光性Gellan gum薄膜之合成與應用 ＊
＊ 名 稱 ＊
＊ ********* ** ＊

執行計畫學生： 黃佳慧
學生計畫編號： NSC 100-2815-C-040-004-E
研 究 期 間 ： 100年07月01日至101年02月28日止，計8個月
指 導 教 授 ： 李明偉

處理方式： 本計畫可公開查詢

執 行 單 位： 中山醫學大學醫學檢驗暨生物技術學系（所）

中華民國 101年03月30日

國科會 100 年度大專生專題研究計畫結案報告

計畫名稱: 感光性 Gellan gum 薄膜之合成與應用

計畫編號: 100-2815-C-040 -004-E

執行單位: 中山醫學大學醫學檢驗暨生物技術學系（所）

指導教授:李明偉 副教授

大專生姓名: 黃佳慧

Photocrosslinkable gellan gum film as the anti-adhesion barrier

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, 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 (Silva-Correia J et al., 2011; Oliveira JT et al., 2010). Gellan gum has also been tested as the drug delivery carrier, cell carrier, wound dressing and guided bone regeneration material (Lee MW et al., 2010; Chang SJ et al., 2010). The stable cross-link 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 polysaccharide can be cross-linked with Ca^{2+} ions, the mechanical properties of polysaccharide are fragile and less malleable (Ichibouji T et al., 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 residue (Powell HM, & Boyce ST, 2006).

To developed a non-toxic method of cross-linking gellan gum and apply in biomedicine is the main purpose of this research work. Crosslinking via photodimerization of polymeric systems has been utilized in various applications. In this study, we designed a new photocrosslinkable gellan gum molecular that contains 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 cinnamate groups presumably as a result of [2+2]π electron cycloaddition (Dong, CM 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 and with anti-inflammatory capacity (Ballabeni, V et al., 2010). In this study, the function of cinnamate not only as a cross-linking agent but also as a

anti-inflammatory drug.

Various kinds of film made of polysaccharides have been reported to use for reducing adhesion formation include Dextran-70, Interceed and Seprafilm™ (Robertson, D et al., 2010), but they are not fully satisfied for the clinical practice. An ideal adhesion prevention product should be resolvable, non-reactive, easy to apply and capable of being fixed in position. In previous study, we have demonstrated gellan gum could prevent fibroblast adhesion and migration. This report also describes the evaluation of the efficacy of photosensitive gellan gum film (denoted as GG-Cin film) in reducing postoperative adhesion formation in the rat model.

2. Methods

2.1. Dissolution of gellan gum in DMSO

To render gellan gum soluble in DMSO, the sodium ions of gellan gum were exchanged with the lipophilic tetrabutylammonium (TBA) ion (Oudshoorn, M. H. M et al., 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 photosensitive group.

2.2. Synthesis of gellan gum-cinnamate (GG-Cin) in DMSO

Gellan gum-TBA was dissolved in DMSO (1% w/w). Cinnamyl bromide was dissolved in DMSO and the concentration was 4% (w/w). A mixture of Gellan gum-TBA solution and cinnamyl bromide solution was stirred at 50℃ for 48 hrs. The mole ratio of cinnamate to gellan gum carboxyl residues

was 5:1. Gellan gum-cinnamate (GG-Cin) product was purified by ethanol precipitation. The purified GG-Cin was analyzed by 1H-NMR (500 MHz, Bruker Advance DRX500).

2.3. Photochemical properties of GG-Cin

The photoreactivity of the GG-Cin was studied by dissolving in DMSO with concentration 0.1% (w/v) and UV light at 252 nm using a mercury lamp（Cole-Parmer 9815-series lamps 100 watts）for different intervals of time. After each irradiation period (2 min), the UV spectra were recorded on scanning spectrophotometer（Milton Roy Spectronic 3000 array）. Crosslinking efficiency was determined by calculating the percent conversion of photoactive chromophore using the following equation (Dong, CM et al., 2005)：

$$\% \text{ crosslinking} = (A_t\text{-}A_0/A_x\text{-}A_\alpha) \times 100$$

where A_0, A_t, and A_α are, respectively, the absorbance values at time 0, time t, and a time after which there was no further change is observed in the absorbance.

2.4. Preparation of GG-Cin film

GG-Cin of 0.2g 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 5cm） and evaporated at 50℃ until the weight of the film was constant. Immersed the film in the ethanol and irradiated with UV light (Cole-Parmer 9815-series Lamps 100 Watts) for 30 min. The cross-linked GG-Cin film was washed with 95% ethanol three times and then dried at room temperature.

2.5. Characterizations of the cross-linked GG-Cin film

An electrical thickness tester (mitutoyo, MDC-25 SB) was used to measure the thickness of the GG-Cin film. We used the FTIR-L396A (Perkin-Elmer) to analyze the properties of the chemical functional groups of the cross-linked GG-Cin film. The analysis of the gel content of the cross-linked GG-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, it 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).

GG-Cin film was cut into 1 cm × 5 cm pieces (Mathew S, & Abraham T. E, 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 GG-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 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 GG-Cin film (diameter: 1.0 cm). Rats of 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 suture 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 days 3 and 7 after surgery to examine the process of adhesion formation at the injured site (Peng, H. H., et al., 2011). Adhesions were scored in a blinded manner according to the method of Zuhlke et al. (Table 1), whereby grade 0 means no adhesions and grade 4 means firm extensive adhesions that are only dissectable 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).

Table 1. Postoperative adhesions grading scale

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)

2.7. Peritoneal fluid analysis

Peritoneal fluids were collected before the operated animals were sacrificed on days 3 and 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 means± standard deviations. For comparison between two groups of data, the Student's t-test was performed. Difference were considered to be statistically significant at $p< 0.05$.

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. The gellan gum-Cin polymer having α,β-unsaturated carbonyl groups in the backbone undergo 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.). It 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 incorporation of the cinnamate group by the presence of methylene proton peaks at 6.3 and 7.5 ppm and a phenyl proton peak at 7.2–7.5 ppm. Conveniently, the degree of cinnamated substitution can be determined by comparing the integrated intensity of the phenyl

and –CH=CH– peaks of the cinnamate group to the integral of the of the $–CH_3$ protons（δ 1.2 ppm）of gellan gum. Accordingly, the degree of cinnamated substitution was about 14.7％ (Chang-Ming Dong et al., 2005).

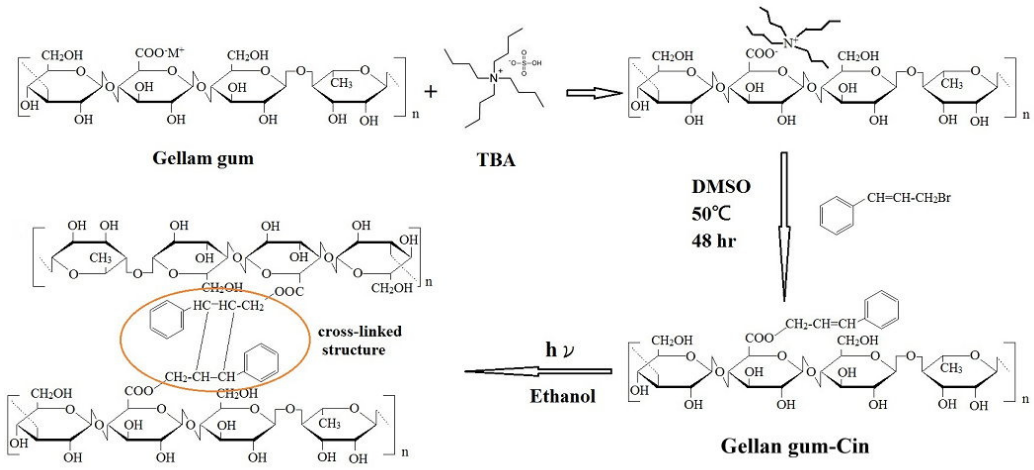


Fig 1.

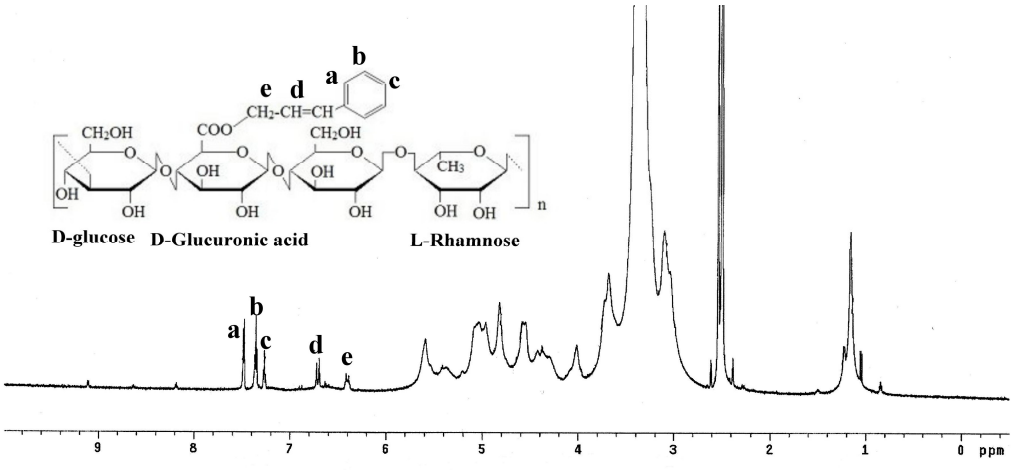
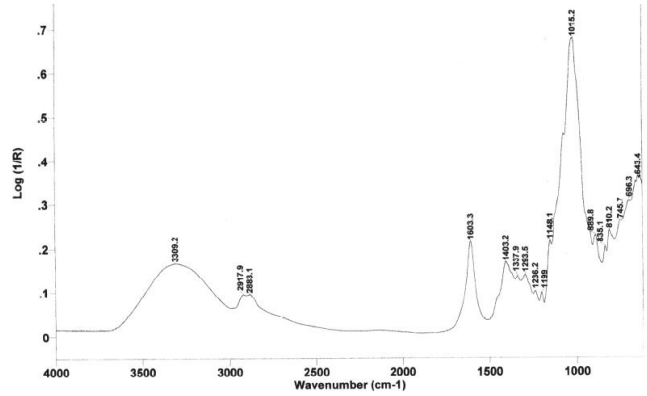


Fig 2.

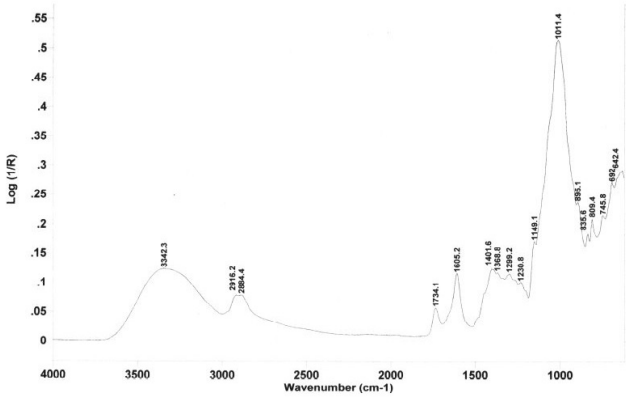
3.2 FTIR characterization of gellan gum-Cin

Fig.3A and 3B 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} for stretching 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 those 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 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 the gellan gum and gellan gum-Cin was appeared a new absorption peak at 1734 cm^{-1}, which was assigned to the benzene group of cinnamate. It also causes the absorption peak of the –OH groups to shift to a higher wave number 3342 cm^{-1}, and the C-O stretching to shift from 1015 to 1011 cm^{-1}. However, the absorption peak of $–CH_2$ groups (2917 cm^{-1}) shows no shift. These data indicate that the new absorption peak is not caused by residual cinnamate and confirms that the covalent grafting reaction was successful.



(A)



(B)

Fig 3.

3.3 Photoreactivity measurements

The UV spectrum of Gellan gum-Cin polymer was shown in Fig 4. As can be seen, the Gellan gum-Cin exhibited an absorption maximum at 252 nm. The spectral changed of the photopolymer solution upon UV irradiation was also shown in Fig 4, the decrease of light absorption at the maximum peak was derived from the conversion of the extend conjugated diene into cyclobutane. Correspondingly, the gradual increase of the number of styryl groups resulted in the increased absorption at 286 nm of Gellan gum-Cin polymer. The photosensitive gellan gum with pendant chalcone groups（α,β-unsaturated carbonyl） undergo a【2+2】photocycloaddiation reaction upon UV are regared as negative-type photoresists (Nagata Minoru, & Inaki Kunihito, 2009; Allen Norman S et al., 1993). The crosslinking efficiency of the gellan gum-cin was increased with light irradiation time and represented in Fig 5. Within 4 min, crosslinking efficiency was 82% and was nearly complete within 16 min. These results suggested that only a short exposure time was enough to induce the cross-linking reaction of the photopolymer.

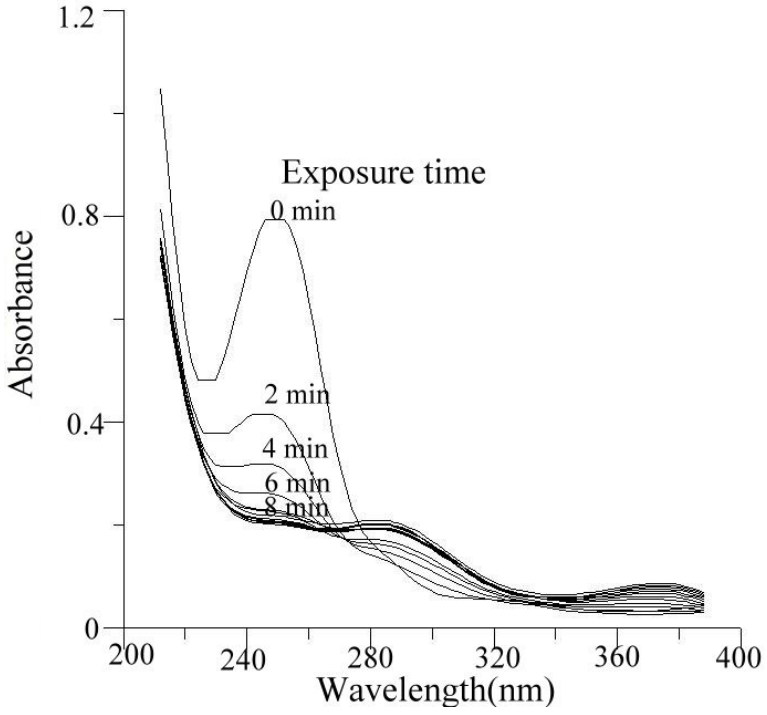


Fig 4：Changes in the UV spectra upon irradiation of Gellan gum-Cin in deionized water.

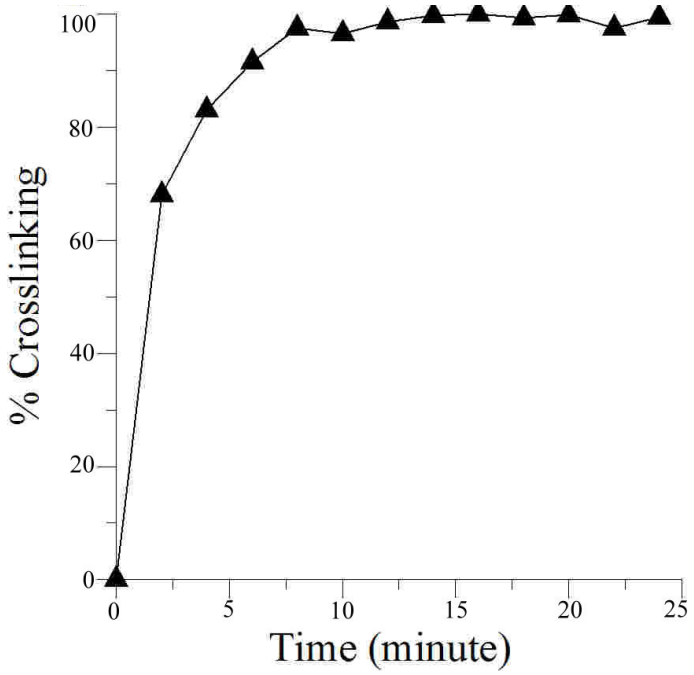


Fig 5：crosslinking efficiency of Gellan gum-Cin polymer with UV irradiation.

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 films was 24 ± 2μm. Gel content of a film is related to the crosslinking density in the film (Nagasawa, N., Yagi, T., Kume, T., Yoshii, F, 2004). The crosslinked gellan gum-cin film have high gel content of about 88 ± 2 %. However, non-cross-linked gellan gum-cin film was rapidly swollen with water. Mechanical testing revealed that the maximum stress and elongation at break of the non-UV irradiation film were 6.6±1.2 N and 8.2%. The maximum stress and elongation at break of the UV crosslinking film were 4.8±1.0 N and 6.8%. It was observed that for higher crosslinking level the film become stiffer. Vijayabaskar et al. (2006), indicated the tensile strength of a polymer is closely correlated to the density of cross-linking. At higher cross-link density, the segments of macromolecules become immobile, due to which system becomes stiff and shows a decrease in elacticity. At present, no standards regarding anti-adhesion film mechanical property are available for clinical evaluation. In this study, the vivo evaluation of crosslinked gellan gum-cin film for the prevention of postoperative, it had sufficient mechanical strength and easy to apply.

3.5 In vivo evaluation of gellan gun-Cin film for the prevention of postoperative sdhesion

The occurrence of tissue adhesion between the cecum and the peritoneum was examined on the 3rd and 7th day after surgery. In the control group (without membrane), the 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 6b). The adhesion scores of the control group were between 3 (severe) and 4 (very severe). On the contrary, gellan gun-cin film effectively prevent tissue adhesion in all rats operated on the day 3 after surgery and reduced adhesion incidences by 60% (3 out of 5 rats operated) on the day 7 after surgery (Fig. 6c and 6d). The adhesion scores of the experimental group were between 0 (no adhesion) and 1 (mild). The superior anti-adhesion capability of the gellan gum-cin film was demonstrated throughout the observation period.

Tissues surrounding the injured sites were dissected and examined. Photomicrographs of the sectioned, H&E stained tissues on the 3th 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 also covered by thick fibrous tissue and contained a thick layer of fibroblast. With gellan gum-cin film treated, 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. Quantitation of the inflammatory cells was performed by a cell-counting method.

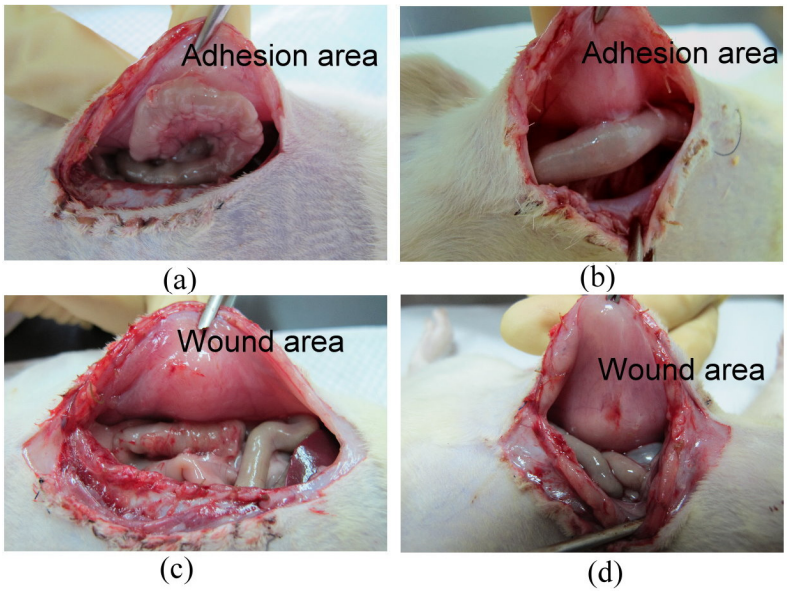


Fig 6.

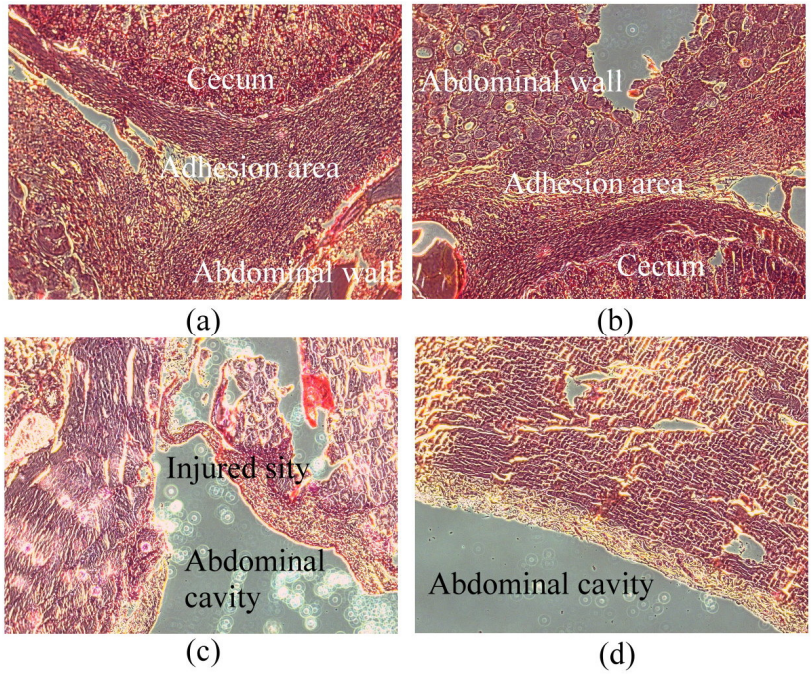


Fig 7.

3.6. Quantitative analysis of the inflammatory cells

We also assessed the number of peritoneal fluid neutrophils in order to evaluate whether the gellan gum-cin had anti-inflammatory capacity. The results showed that on the 3rd and 7th day 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.

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. For all test groups, the numbers of neutrophils reached a maximum within the first 3 days after surgery abd then gradually decreased over the 7 day period of observation. On the 3th day after surgery, the numbers 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 numbers of neutrophils in the control group was 1.88 (P-value 0.0008) times that of the experimental group. Neutuophils 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 up the damaged tissues. However, if an injury is not properly addressed inflammation and scar tissue (adhesion fibrosis) can become more severe, leading to the beginning of the chronic injury cycle (Delavary, B. M et al., 2011). Our results 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.

Conclusion

In this work, we have successfully cross linked the gellan gum moleculars with cinnamate groups and demonstrated gellan gum-cin molecular with anti-adhesion capacity and has great potential for future use in clinical application.

Reference

- Jansson, P. E., Lindberg, B. & Sandford, P. A. (1983). Structural studies of gellan gum an extracellular polysaccharide elaborated by Pseudomonas elodea. Carbohydrate Polymers, 124, 135–139.
- O'Neil, M. A., Selvendran, R. R., & Morris, V. J. (1983). Structure of the acidic extracellular gelling polysaccharide produced by Pseudomonas elodea. Carbohydrate, 124, 123-133.

- Silva-Correia J, Oliveira JM, Caridade SG, Oliveira JT, Sousa RA, Mano JF, Reis RL. Gellan gum-based hydrogels for intervertebral disc tissue-engineering applications. J Tissue Eng Regen Med. 2011 Jun;5(6):e97-107
- Oliveira JT, Martins L, Picciochi R, Malafaya PB, Sousa RA, Neves NM, Mano JF, Reis RL. Gellan gum: a new biomaterial for cartilage tissue engineering applications. J Biomed Mater Res A. 2010 Jun 1;93(3):852-63.
- Lee, M. W., Chen, H. J., & Tsao, S. W. (2010). Preparation, characterization and biological properties of Gellan gum films with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide cross-linker. Carbohydrate Polymers, 82(3), 920–926.
- Chang, S. J., Kuo, S. M., Liu, W. T., Niu, C. G., Lee, M. W., & Wu, C. S. (2010). Gellan gum films for effective guided bone regeneration. Journal of Medical and Biological Engineering, 30, 99–103.
- Ichibouji, T., Miyazaki, T., Ishida, E., Sugino, A., Ohtsuki, C. (2009). Apatite mineralization abilities and mechanical properties of covalently cross-linked pectin hydrogrls. Mater Sci Eng C, 29(6), 1765-1769.
- Powell, H. M., Boyce, S. T. (2006). EDC cross-linking improves skin substitute strength and stability. Biomaterials, 27(34), 5821-5827.
- Dong, C. M., Wu, X., Caves, J., Rele, S. S., Thomas, B. S., Chaik, E. L. (2005). Photomediated crosslinking of C6-cinnamate derivatized type I collagen. Biomaterials, 26 (18), 4041-4049.
- Ballabeni, V., Tognolini, M., Giorgio, C., Bertoni, S., Bruni, R., Barocelli, E. (2010). Ocotea quixos Lam. essential oil: In vitro and in vivo investigation on its anti-inflammatory properties. Fitoterapia, 81 (4), 289-295.
- Robertson, D., Lefebvre, G., Leyland, N., et al. (2010). Adhesion prevention in gynaecological surgery. J Obstet Gynaecol Can, 32(6), 598-608.
- Oudshoorn, M. H. M., Rissmann, R., Bouwstra, J. A., Hennink, W. E. (2007). Synthesis of methacrylated hyaluronic acid with tailored degree of substitution. Polymer, 48 (7), 1915-1920.
- Mathew, S., Abraham, T. E. (2008). Characterisation of ferulic acid incorporated starch–chitosan blend films. Food Hydrocolloids, 22 (5), 826-835.
- Peng, H. H., Chen, J. W., Yang, T. P., Kuo, C. F., Wang, Y. J., Lee, M. W. (2011). Polygalacturonic acid hydrogel with short-chain hyaluronate cross-linker to prevent postoperative adhesion. Journal of Bioactive and Compatible Polymers, 26 (6), 552-564.
- Zuhlke, H. V., Lorenz, E. M., Savvas, V. (1990). Pathophysiology and classification of adhesions. Langenbecks Arch Chir Suppl II Verh Dtsch Ges Chir, 190, 1009-1016.
- Coutinho, D. F., Sant, S.V., Shin, H. Oliveira, J. T., Gomes, M.E., Neves, N. M., Khademhosseini, A., Reis, R. L. (2010). Modified Gellan Gum hydrogels with tunable physical and mechanical properties. Biomaterials, 31 (29), 7494-7502.
- Sudhamani, S. R., Prasad, M. S., & Udaya Sankar, K. (2003). DSC and FTIR studies on gellan and polyvinyl alcohol (PVA) blend films. Food Hydrocolloid, 17, 245–250.

- Nagata, M., Inaki, K. (2009). Synthesis and characterization of photocrosslinkable poly(l -lactide)s with a pendent cinnamate group European Polymer Journal, 45 (4), 1111-1117.
- Allen, N. S., Mallon, D., Timms, A., Green, A. W., Catalina, F. (1993). Synthesis and spectroscopic properties of novel cinnamate derivatives of thioxanthone: photocuring activity versus photodimerization. Polymer, 34 (11), 2401-2406.
- Nagasawa, N., Yagi, T., Kume, T., Yoshii, F. (2004). Radiation crosslinking of carboxymethyl starch. Carbohydrate Polymers, 58 (2), 109-113.
- Vijayabaskar, V., Tikku, V. K., Bhowmick, & Anil, K. (2006). Electron beam modification and crosslinking: Influence of nitrile and carboxyl contents and level of unsaturation on structure and properties of nitrile rubber. Radiation Physics and Chemistry, 75, 779–792.
- Delavary, B. M., van der Veer, W. M., van Egmond, M., Niessen, F. B., Beelen, R. H. J. (2011). Macrophages in skin injury and repair . Immunobiology, 216 (7), 753-762.