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Original Article

Synthesis and Characterization of Cinnamated Polygalacturonic Acid for Biomedical Application

Shih-Hsien Chang[1], Kai-Sung Wang[1], Weng-In Leong[2], Ming-Wei Lee[2,3*]

[1] School of Public Health, Chung Shan Medical University, Taiwan
[2] School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taiwan
[3] Department of Clinical Laboratory, Chung Shan Medical University Hospital, Taiwan

Polygalacturonic acid (PGA) with varying degrees of cinnamated subsititution were synthesized. 6.2-15.1% of the D-galacturonic residues of the cinnamated PGA (PGA-Cin) reacted with cinnamate groups. An investigation of the photochemical properties revealed that the photoconversion rate and rate constant of PGA-Cin was 80% and $5.95\times10^{-3}s^{-1}$ after UV irradiation. The anti-adhesion films prepared from PGA-Cin exhibited high gel content and were found to efficacious in inhibiting bacterial growth. We evaluated their biocompatibility and anti-adhesion capability in both *in vitro* and *in vivo* experiments. The PGA-Cin films did not show any evidence of cytotoxic effects, as they did not induce any significant increase of cytoplasmic LDH release from the L929 cells in contact with them. When implanted into rats, the PGA-Cin films (anti-adhesion incidence 80.9%) exhibited more anti-adhesion potential than the commercial product Seprafilm™ (anti-adhesion incidence 47.6%).

Key words: polygalacturonic acid, cinmamate, photocrosslink, anti-adhesion

Introduction

Pectic polysaccharides exist in plant cell walls and consist of mainly of galacturonic acid residues linked together by α-1-4 glycosidic linkages. Pectic polysaccharides with methyl ester groups are known as pectin. Those with free acid groups neutralized with ions are known as polygalactruonic acid (PGA)[1]. PGA is widely used for biomedical and pharmaceutical applications owing to its excellent biocompatibility and biodegradability[2,3]. In this study, we synthesized and characterized cinnamated-PGA biomacromolecules and then evaluated their biocompatibility, antibacterial activitive and anti-postsurgical adhesion potential to further evaluation possible applications. Postsurgical abdominal adhesions are urgent problems in surgery today. Intra-abdominal adhesion formation is the result of both insufficient fibrinolytic capacity and increased fibrin exudates[4,5]. Normal fibrinolytic activity prevents fibrinous attachments after surgery, and mesothelial repair occurs within 5 days of trauma. During this period, a single cell layer of new peritoneum covers the wound area, replacing fibrinous exudates. However, if fibrinolysis activity is suppressed, fibroblasts will migrate, proliferate and form adhesion tissue. The inflammatory system plays a major role in the maintenance of the fibrinogenic/fibrinolytic balance[6]. Inflammation is a response of a tissue to injury, and severe inflammation activates antithrombin III and increases fibrin formation.

* Corresponding Author: Ming-Wei Lee
Address: No.110, Sec. 1, Jianguo N.Rd., Taichung 402, Taiwan, R.O.C.
Tel.: +886-4-24730022 ext. 12412
Fax: +886-4-23248171
E-mail address: d880430@csmu.edu.tw

Inflammation also activates PAI-I (plasminogen activator inhibitor 1) when the fibrinolysis system is insufficient[7].

Crosslinking via photodimerization of polymeric systems has been utilized in various applications. In this work, we used cinnamate derivative as the cross-linking agent to prepare photosensitive PGA anti-adhesion film (denoted PGA-Cin film). 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[8,9]. The reaction does not require the addition of a light sensitive initiator, and crosslinking upon irradiation with UV light also can inhibit the formation of bacterial colony. Cinnamate is a natural tropane alkaloid, which is found within the Erythroxylum coca plant, possesses anti-inflammatory and non-toxic properties[10,11]. In this study, cinnamate functions not only as a cross-linking agent but also as an anti-inflammatory drug.

Drugs and physical barriers have been used for several years to reduce the occurrence of tissue adhesion years[12]. It is believed that early inflammatory reaction of the injured wound results in a series of repair behaviors leading to a greater tendency toward tissue adhesion. When used as physical barrier, film should remain between the injured surfaces for at least 36hr for the optimal reduction of the adhesions[13]. To maximize the reduction of adhesion, a combination of physical barrier and anti-inflammatory drug has been proposed. Therefore, the goal of this study was to develop a new anti-adhesion film based on PGA-Cin for use in surgery. The PGA-Cin film was characterized and its performance evaluated and compared with Seprafilm™, a commercial product used clinically.

Methods

Synthesis of cinnamated polygalacturonic acid (PGA-Cin)

Polygalacturonic acid (Sodium salt) was dissolved in 85-90°C of deionized water (DDW). The concentration and pH of the PGA solution were 1.0 (w/v %) and 6.4, respectively. Cinnamyl bromide was dissolved in DMSO (Dimethyl sulfoxide) at a concentration of 4.0 (w/v %). A mixture of PGA solution and cinnamyl bromide solution was stirred at room temperature for 96 hrs. Three mole ratios of cinnamate to PGA carboxyl residues were investigated (0.5:1, 1:1 and 1.5:1). The resulting PGA derivatives were designated PGA-CinR0.5, PGA-CinR1.0, and PGA-CinR1.5, respectively. The solutions were purified by precipitation in 4-fold volume of ethanol and then dissolved in deionized water. 1H-NMR (500 MHz, Bruker Advance DRX500) and UV-vis (Milton Roy Spectronic 3000 array) spectroscopy were used to monitor the incorporation of cinnamate groups.

Photochemical properties

The photoreactivity of the PGA-Cin polymers was studied by dissolving samples in deionized water at a concentration of 0.1 (w/v %) and then exposing them to UV light at 252 nm using a mercury lamp (Cole-Parmer 9815-series lamps 100 watts) for different time intervals. After each irradiation period (2 min), UV spectra were recorded on a scanning spectrophotometer (Milton Roy Spectronic 3000 array). The extent of photocrosslinking was then determined from the decrease in UV absorption intensity at 252 nm using the following equation[14]:

$$\text{Extent of conversion (\%)} = (A_0\text{-}At/A_0) \times 100$$

Where A0 and At are the absorbance values at time 0 and time t, respectively.

Preparation of PGA-Cin film

Approximately 20 ml of aqueous PGA-Cin solution (2% w/v) was poured onto a glass dish (diameter 10 cm) and evaporated at 50°C until the weight of the film was constant. The film was then immersed in DMF (dimethyl fumarate) for 24 h and then irradiated with UV light for 12 min. The cross-linked film was washed with 95% ethanol three times and dried at room temperature.

Characterization of the PGA-Cin films

The PGA-Cin films were analyzed using an FTIR-L396A (Perkin-Elmer) spectrophotometer. The gel content of the various PGA-Cin films were analyzed following a previously described procedure[15]. Briefly, a cross-linked film was

weighed after drying (W1) and then swelled in phosphate buffered saline at 37°C for 24 h. After that, the same film was dried in a vacuum oven for 12 h at 60°C and then reweighed (W2). The gel content was calculated using the following equation:

$$\text{Gel content (\%)} = (W_2/W_1) \times 100\%:$$

Cytotoxicity test

The cytotoxicity of the anti-adhesion film (PGA-CinR1.5) was determined from the amount of cytoplasmic lactate dehydrogenase (LDH) released by cells incubated with the polymeric film. First, the PGA-CinR1.5 films were immersed in cell culture medium in 48-well tissue culture plates. Next, aliquots of 400 ml L-929 fibroblast cell suspensions were added to each well at a density of 4×10^4 cells/ml and incubated at 37°C. After a designated incubation period, the medium was aspirated and centrifuged at 250 g for 10 min. Supernatant (100 ml) was taken from each well, mixed with 100 ml of the reagent (LDH-Cytotoxicity Assay Kit, BioVision), and then incubated in a 96-well plate for 30 min at room temperature. The absorbance of the reaction mixture at 490–500nm was measured using a microtiter plate reader with reference wavelength at 600 nm. The amounts of LDH released from the cells cultured on a 48-well plate were used as the background release, whereas those amounts released from the cells lysed with 1% Triton X-100 were used as positive controls. The cytotoxicity (%) of the sample was calculated using the following equation[16] with four independent experiments being conducted:

Cytotoxicity (%)
= 【(test sample-background release) / (positive control-background release)】×100%

Antibacterial test

In the antibacterial assay, 24 pieces of dry PGA-CinR1.5 films (1 cm^2) were prepared and then irradiated with UV light for 12 min. PGA-CinR1.5 films without UV irradiating were used as controls. These films were placed on the sterile LB agar plate (GIBCOBRL) and then aerobically incubated at 37℃. After 24h, the bacterial growth incidence was assessed (the number of bacterial growth on PGA-CinR1.5 films/the number of samples).

Animal implant study

A total of 63 Sprague-Dawley rats (200 – 250 g) were operated on 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 blade until the serosal surface was disrupted, though the hemorrhagic wall not perforated. The denuded abdominal wall was then covered with either PGA-CinR1.5 film (diameter 1.5 cm) or Seprafilm™ (diameter 1.5 cm). Rats in the control group were not covered with any anti-adhesion film. Contact between the cecum and opposing abdominal wall was maintained in all animal groups with two non-occluding loops of 4/0 polypropylene suture placed 2 cm apart. After completion of the procedure the abdomen was closed in double layer using 4/0 polypropylene in a continuous fashion. The experimental rats were sacrificed at 3, 7, and 14 days after surgery to examine the process of adhesion formation at the injured site. The anti-adhesion incidence was expressed as a ratio of number of rats with adhesion to number of rats receiving operations.

Peritoneal fluid analysis

Peritoneal fluids were collected before the animals were sacrificed on the 3rd, 7th, and 14th day after surgery. The peritoneal fluid was aspirated through a pipette with a bulb tip after 2 ml of the Dulbecco's modified Eagle's medium containing heparin was injected into the peritoneal cavity. The amounts of neutrophils and monocytes in the collected fluid were determined by counting cell using a standard clinical hemocytometer (ADVIA 120, Bayer).

Statistical analysis

Each of the experiments was repeated at least five times. For comparison between two groups of data, Student's t-test was performed. Differences were considered to be statistically significant when $p< 0.05$.

Results

The various PGA-Cin polymers were analyzed by 1H-NMR (Fig 1). The characteristic peak of cinnamate (aromatic protons) at δ = 7.39-7.53 ppm was present in the spectra of the photosensitive PGA polymer. The peak for the PGA molecule was at δ = 3.15-5.35 ppm[17]. The degree of cinnamated substitution could be conveniently determined by comparing the integrated intensity of the cinnamate group with the integral of the aliphatic protons (δ = 4.89) of PGA. We found the degree of cinnamated substitution to vary from 6.2 % to 15.1 % as we changed the molar ratio of cinnamate to PGA carboxyl groups from 0.5:1 to 1.5:1. The absorption coefficient ($\varepsilon = 6.78\times10^{3}M^{-1}cm^{-1}$) of the model compound D-Gluconic acid-cinnamate could also be used to determine the degree of cinnamate substitution from the absorption spectrum of the photosensitive polymer. Table 1 shows the the PGA-Cin materials and their corresponding cinnamate contents. The degrees of cinnamate substitution determined by 1H-NMR were very close to those calculated from the UV absorption spectra. These findings suggest that it was possible to prepare water-soluble cinnamated polygalacturonic acid in a 5:1 water-DMSO mixture.

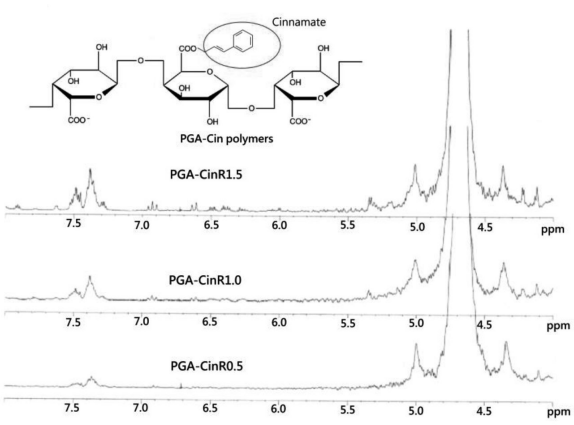

Fig 1. ^{1}H-NMR spectrum of various PGA-Cin polymers.

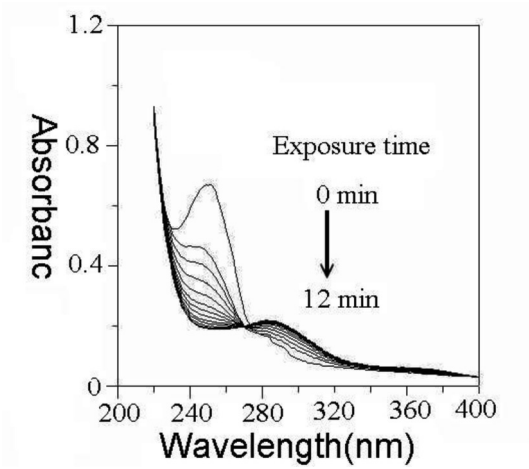

Fig 2. The change of UV spectra of the PGA-CinR1.5 induced by irradiation of UV light through the filter.

The UV spectrum of the PGA-CinR1.5 polymer is shown in Fig 2. The PGA-Cin polymer exhibited an absorption maximum at 252 nm. The spectral changes of the photopolymer when UV irradiated can also seen in Fig 2. The decrease in light absorption at the maximum peak was due to the conversion of the extended conjugated diene into cyclobutane[8,9]. The gradual increase in the number of styryl groups resulted in an increase in absorption at 287 nm[8,9]. The rate of disappearance of the chalcone double bond upon UV irradiation is presented in Fig 3. The photoconversion rate increased with light irradiation time. Approximately 80% of the various PGA-Cin polymers were photoconverted after 12 min of UV irradiation. These results suggest that a short exposure time can induce cross-linking of the photopolymer.

In the absorption change process, the crosslinking rate constant (k) could be determined

Table 1. Characterization of PGA-Cin polymers

	Cin / COOH (molar ratio)	cinnamate content (mol%) ^{1}H-NMR	cinnamate content (mol%) -UV	Yield (%)
PGA-CinR0.5	0.5:1	6.2%	5.3%	59
PGA-CinR1.0	1:1	9.4%	8.9%	71
PGA-CinR1.5	1.5:1	15.1%	14.1%	78

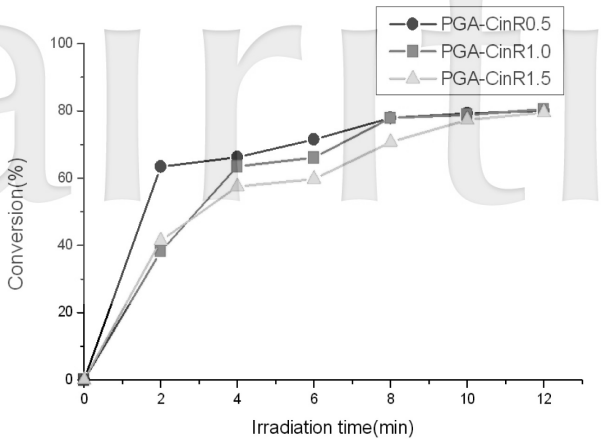

Fig 3. Rate of disappearance of pendant chalcone double bond of PGA-Cin polymers with UV irradiation.

the follow equation: $At/ A_0 = A_0exp(-kt) + Ar$ (Ar: the residual absorbance at $t = \infty$)[18,19]. The first-order plots from this equation for PGA-CinR0.5, PGA-CinR1.0 and PGA-CinR1.5 are shown in Fig 4. The rate constant for PGA-CinR0.5 and PGA-CinR1.0 were the same ($5.95\times10^{-3}s^{-1}$), and that for PGA-CinR1.5 was slightly less ($5.32\times10^{-3}s^{-1}$). These findings suggest that the photocrosslink of PGA-CinR1.5 slowly results in steric hindrance.

LDH is a stable enzyme present in the cytosol released during cell lysis. Fig 6 shows the cytotoxic effects of the PGA-CinR1.5 film after different

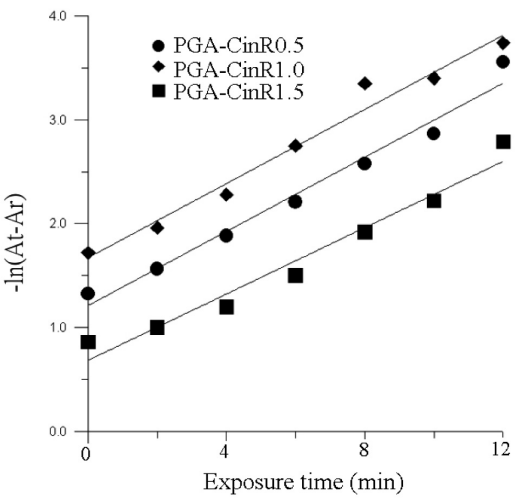

Fig 4. Plot of –ln (At-Ar) as a function of time according to equation ($At/ A_0 = A_0exp(-kt) + Ar$) for the PGA-Cin polymers.

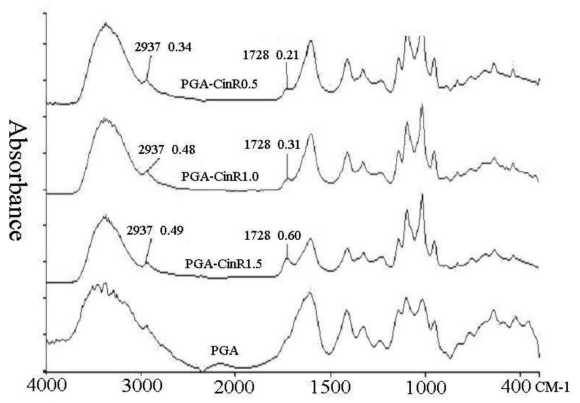

Fig 5. FTIR spectra of PGA and various PGA-Cin polymers.

exposure times expressed as the percentage of LDH released into the culture medium. When the PGA-CinR1.5 films were in contact with L929 cells for 3, 12, or 24 h, the cytotoxicity induced was less than 20%. We found no significant difference in the control group (culture plate) with regard to LDH release, which indicated that PGA-CinR1.5 films were non-toxic to cells.

In our case, the PGA-Cin films were crosslinked via short wavelength UV irradiation (252 nm). Short wavelength ultraviolet light has an effective antibacterial effect. We performed the antibacterial assay to examine the antibacterial properties of PGA-Cin films before and after UV irradiation. Bacterial colonies formed on UV irradiated and non-UV irradiated films are shown in Fig 7. In the non-UV irradiated films, bacteria

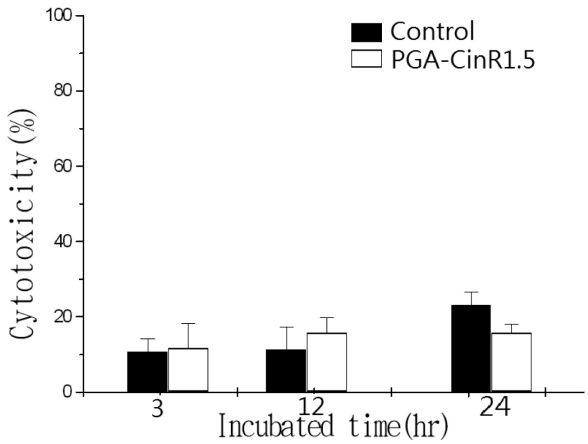

Fig 6. Cytotoxicity of PGA-CinR1.5 film.

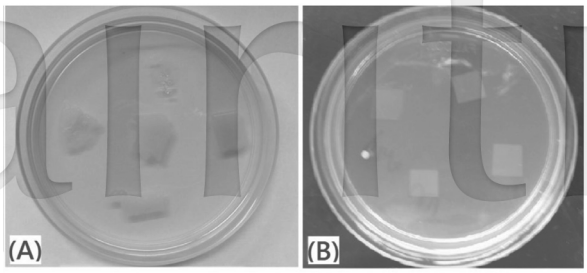

Fig 7. Antibacterial results of PGA-CinR1.5 film before (7A) and after (7B) UV irradiation.

grew all areas of the film surface. The incidence rate for bacterial colony formation in the non-UV irradiated film group was 14/24 (58%), which was significantly higher than that of the UV irradiated film group (0/24). These results indicate that the PGA-Cin films that were UV-crosslinked had an antibacterial effect and could be used clinically.

We evaluated the anti-adhesion efficacy of the PGA-Cin films in rats. The occurrence of tissue adhesion between the cecum and the peritoneum was examined on the 3rd, 7th, and 14th day after surgery. Tissue adhesion data for the three test groups are summarized in Table 2. The incidences in adhesion in the uncovered and commercial film-covered (Seprafilm™) denuded abdominal walls were 18/21 and 11/21, respectively. Notably, the PGA–CinR1.5 films prevented tissue adhesion in 17 of 21 rats. Macroscopic views of the surgical site for the three test groups after 14 days are shown in Fig 8A-B-C. The site treated with PGA-CinR1.5 was free of dense adhesion tissue and appeared clear compared with the control and SeprafilmTM-treated groups. Histological photomicrographs of the tissue sections on the 14th day of repair are shown in Fig 8D-E-F. For

Table 2. Formation of post-surgical tissue adhesion of the rats operated

	3 days	7 days	14 days	Combined data	anti-adhesion incidence
Control	7/7*	6/7	5/7	18/21	14.2%
PGA-CinR1.5	1/7	2/7	1/7	4/21	80.9%
Seprafilm™	3/7	4/7	4/7	11/21	47.6%

Note: *number of rats with adhesion/number of rats operated

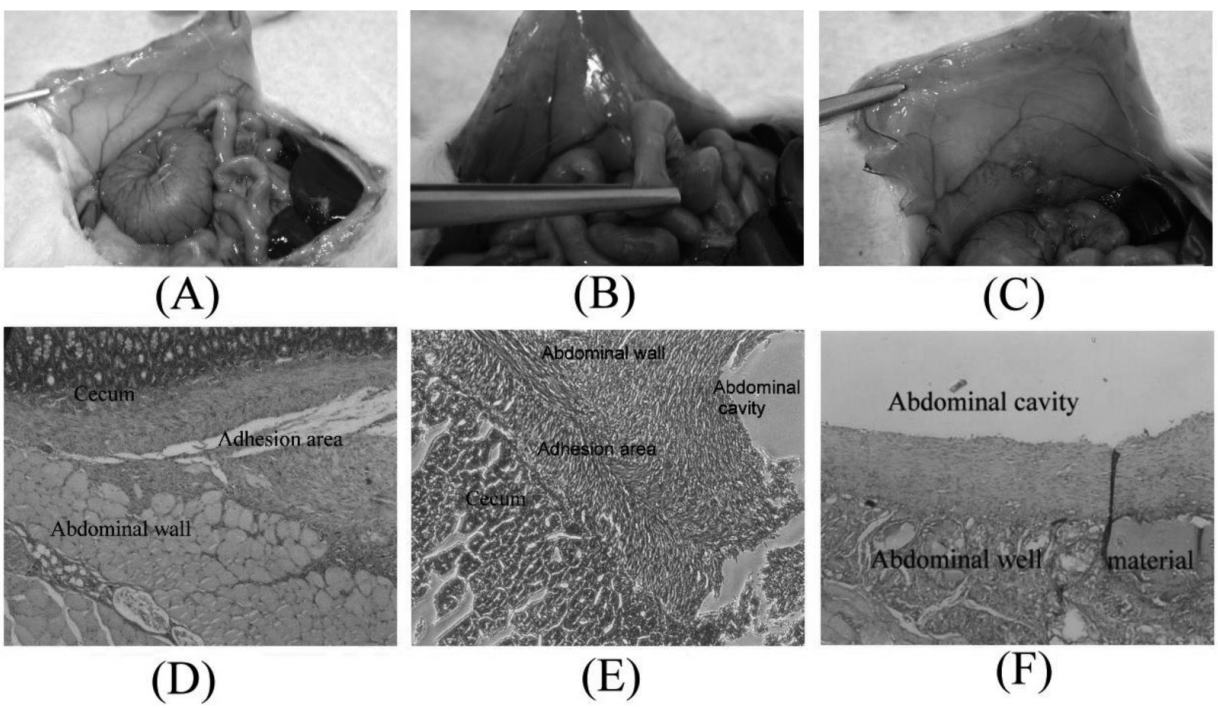

Fig 8. Macroscopic views and photomicrographs (HE stain,100x) of the wound site in rats 14 days after surgery, (A,D) control group, (B,E) treated with SeprafilmTM, (C,F) treated with PGA-CinR1.5 film.

the control group, a newly formed adhesive tissue containing a thick layer of fibroblasts was found between the peritoneal wall and the mucosa of the cecum. The connected area also contained infiltrating polymorphonuclear leukocytes. For the Seprafilm™ group, the adhesion area was thick and dense compared with the control animals on the 14th day after surgery. However, the inflammatory cells were not apparent. In addition, the material film had entirely degraded. In the case of the PGA-treated animals, the peritoneal wall inner surface was smooth and clear without adhesion formation or cellular infiltration. We found PGA to still be present on the 14th day after surgery and to persist for 2 months *in vivo*. These results show that PGA–Cin films have superior anti-adhesion capabilities and do not induce moderate or serious inflammatory reactions compared with the untreated groups and Seprafilm™ treated groups.

We used a hemocytometer to count the number of peritoneal fluid neutrophils and monocytes elicited by the PGA-CinR1.5 film and Seprafilm™. In the early period after injury (up to 3 days), the most common leukocytes in the peritoneal lesions were neutrophils. These cells were derived from the acute inflammatory reaction. Neutrophils are involved in the pathophysiology of intraperitoneal adhesion formation[21]. Changes in the number of peritoneal fluid neutrophils for the three test groups are shown in Fig 9A. For all test groups, the numbers of neutrophils reached a maximum within the first 3 days after surgery, before gradually decreasing over the 14-day observation period. In the PGA-treated animals, the number of neutrophils increased slightly on the 3rd day but was not significantly different from the control group (*P* value 0.057). These results indicate that the PGA film and Seprafilm™ did not elicit significant acute inflammatory reactions compared with the control group.

We also assessed the number of peritoneal fluid monocytes to evaluate whether the degraded anti-adhesion materials elicited inflammatory reactions. Monocytes are attracted to foreign material as a result of chemotactic signals pertaining to the inflammatory process[22]. The numbers of peritoneal fluid monocytes in the three test groups are shown in Fig 9B. More monocytes were found in the case of Seprafilm™-treated animals compared with the control group after surgery (*P* value 0.031 and 0.033 for 3 and 14 days, respectively). The number of monocytes found in the PGA-film treated rats also increased significantly compared with the control group (*P* value 0.036 and 0.042 for days 3 and 14, respectively). From these results, it can be inferred that the degradation of the PGA-Cin film and Seprafilm™ began to elicit an inflammatory reaction in the early period after injury.

Discussion

A stable PGA film can be obtained by forming bonds between PGA molecular chains and chemical

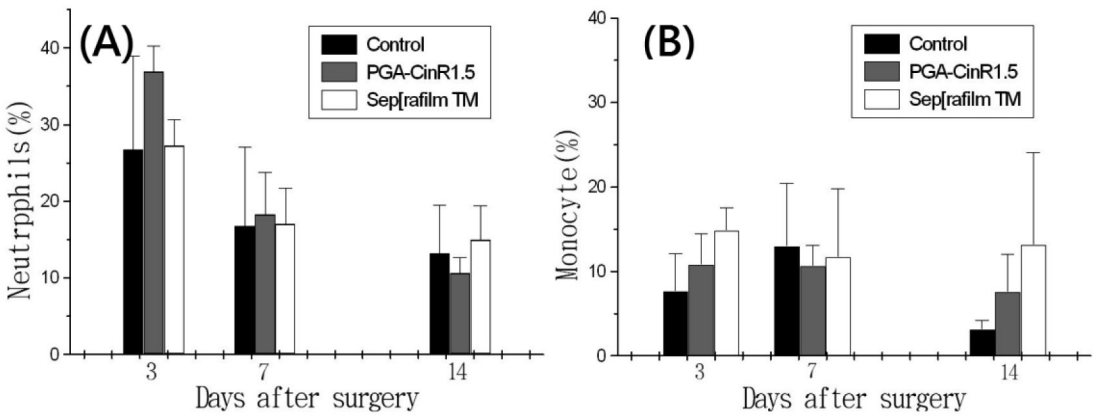

Fig 9. The population of neutrophils (A) and monocytes (B) (expressed in % number) in peritoneal fluid on the 3rd, 7th and 14th days after surgery (n=5, results are given as mean values, bars represent standard deviation).

cross-linkers. There have been several chemical reagents for the cross-linking of PGA, including 1,3-bis (3-chloro-2-hydroxypropyl) imidazolium hydrogen sulphate (BCHIHS), epichlorohydrin and dialky halides[13]. However, chemical cross-linkers can be cytotoxic depending on dosage response and cross-linker residue. In this work, we synthesized photosensitive PGA and prepared PGA-Cin films. The advantages of these photosensitive materials include delayed PGA degradation, antibacterial activity and fast crosslinking times. In addition, the photo-crosslink reaction did not require the addition of a light-sensitive initiator. Cinnamate is a natural tropane alkaloid that possesses anti-inflammatory and non-toxic properties. Photosensitive polymer synthesis is generally performed in organic solvents. In this study, we used a 5:1 mixture of water-DMSO to prepare water-soluble cinnamated polysaccharides without transferring them to the tetrabutylammonium (TBA) salt form[23] as previously reported. Three molar ratios of cinnamate to PGA carboxyl residues were investigated (0.5:1 (PGA-CinR0.5), 1:1 (PGA-CinR1.0) and 1.5:1 (PGA-CinR1.5)), and two methods were used to determine the degree of cinnamate substitution. The results from the 1H-NMR spectra were in accordance with the UV absorption spectra data and confirmed that the water-DMSO mixture could be used as a solvent for photocuring.

The photosensitivity of the PGA polymers was studied in water by irradiating them under a high pressure Hg lamp. PGA-Cin exhibited an absorption maximum at 252 nm (C = C bond). The peak decreased with irradiation time from the conversion of the extended conjugated diene into cyclobutane[24]. A gradual increase in the number of styryl groups resulted in increased absorption at 287 nm. The photosensitive PGA with pendant chalcone groups (α, β-unsaturated carbonyl) underwent a 【2+2】 photocycloaddition reaction when irradiated with UV in much the same way as negative-type photoresists[25]. Kinetic evaluation of the photocrosslinking of PGA-CinR0.5, PGA-CinR1.0 and PGA-CinR1.5 indicated that these photopolymers were not different in steric hindrance, with similar rate constants of approximately $5.95\times10^{-3}s^{-1}$. The photoconversion rate reached 80% for the various PGA-Cin polymers after 12 min of UV irradiation. Thus, PGA-Cin polymers are highly photosensitive and can crosslink quickly after UV irradiation.

In this study, we designed a novel anti-adhesion film based on the PGA-CinR1.5 polymer and evaluated its biocompatibility and post-surgery anti-adhesion capability. Various biodegradable films based on polysaccharides have been developed for anti-adhesion including Interceed and Seprafilm™ [26,27]. Polysaccharides have high amounts of free carboxyl groups, and their pKas are generally below 7.0. At a neutral pH, these polysaccharides and living cells are negatively charged. Strong negative charges on the polysaccharide film surfaces cause long-range electrostatic repulsion that can prevent adhesion of cells[28]. The details of cell-polysaccharide interaction, however, remain largely uncharacterized. In this work, we demonstrated that PGA-Cin films are biocompatible and antibacterial. The PGA-Cin films exhibited the most promising anti-adhesion potential *in vivo* (anti-adhesion incidence 17/21) compared with the commercial product Seprafilm™ (anti-adhesion incidence 10/21). Therefore, our study suggests that photosensitive polygalacturonic acid has potential as an anti-adhesion biomaterial.

Conclusions

In this work, we have successfully cross linked the PGA moleculars with cinnamate groups and demonstrated that this PGA-Cin molecular has good biocompatibility. The result of our anti-adhesion evaluation suggests that it has great potential in biomedicine.

Acknowledgments

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Original Article

聚半乳醣醛酸偶合肉桂之合成、定性與生醫應用

張時獻[1] 王凱淞[1] 梁詠賢[2] 李明偉[2,3*]

[1] 中山醫學大學 公共衛生學系
[2] 中山醫學大學 醫學檢驗暨生物技術學系
[3] 中山醫學大學附設醫院 檢驗科

本研究合成聚半乳糖醛酸（Polygalacturonic acid，PGA）與不同比例肉桂（cinnamate）偶合之產物（以PGA-Cin表示）。聚半乳糖醛酸上的官能基團之肉桂接枝率介於6.2-15.1%。研究肉桂修飾之聚半乳糖醛酸的光化學特性，證實其光吸收係數（ε）為$6.78\times10^{3}M^{-1}cm^{-1}$。肉桂修飾之聚半乳糖醛酸經由紫外線照射之光交聯反應，光交聯轉換率及光交聯速率常數分別為80%和$5.95\times10^{-3}s^{-1}$。將肉桂修飾之聚半乳糖醛酸製成薄膜並應用於腹腔手術後之抗組織沾黏。此抗沾黏薄膜具有高的含膠量以及抗微生物生長之特性。藉由體內、體外研究測試薄膜的生物相容性以及抗組織沾黏效能。研究證實，肉桂修飾之聚半乳糖醛酸薄膜並不會引發L929纖維母細胞死亡，並引起細胞質釋放大量LDH，顯示薄膜為無毒性。當作為動物活體之抗組織沾黏測試，肉桂修飾之聚半乳糖醛酸薄膜抗沾黏效率達80.9%，遠超過市售的抗沾黏商品Seprafilm™（抗沾黏效率為47.6%）。因此，本研究開發之聚半乳糖醛酸薄膜，未來具有臨床應用的價值。

關鍵詞：聚半乳糖醛酸、肉桂、光交聯、抗沾黏

* 通訊作者：李明偉
通訊地址：台中市402建國北路一段110號
聯絡電話：04-24730022 分機12412
電子信箱：d880430@csmu.edu.tw