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Injectable pullulan hydrogel for the prevention of postoperative tissue adhesion

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ABSTRACT

Methods for reducing and preventing postoperative abdominal adhesions have been researched for decades; however, despite these efforts, the formation of postoperative peritoneal adhesions is continuously reported. Adhesions cause serious complications such as postoperative pain, intestinal obstruction, and infertility. Tissue adhesion barriers have been developed as films, membranes, knits, sprays, and hydrogels. Hydrogels have several advantages when used as adhesion barriers, including flexibility, low tissue adhesiveness, biodegradability, and non-toxic degraded products. Furthermore, compared with preformed hydrogels, injectable hydrogels can fill and cover spaces of any shape and do not require a surgical procedure for implantation. In this study, pullulan was modified through reaction with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to introduce carboxyl and phenyl groups as crosslinking sites. The grafting of tyramine on pullulan allows crosslinking branches on pullulan backbone. We successfully fabricated pullulan hydrogel with an enzymatic reaction using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). The chemical structure of modified pullulan was analyzed with ATR-FTIR and ^{1}H NMR spectroscopies. Rheological properties were tested by measuring storage modulus with varying H_2O_2, HRP, polymer solution concentrations and tyramine substitution rates. Cell viability and animal tests were performed. The modified pullulan hydrogel is an invaluable advance in anti-adhesion agents.

1. Introduction

Decades of research have been devoted to studies of biocompatible materials for biomedical applications, and biomaterials combining synthetic and natural polymers have shown considerable promise. Surgical operations *via* peritoneal incision cause serious problems such as female infertility, chronic pelvic pain, and small bowel obstruction [1]. Tissue adhesions occur in more than 93% of patients after laparotomy [2]. Various methods for preventing abdominal adhesions have been implemented, including the use of a thermo-responsive polymer (poly[*N*-isopropylacrylamide]-grafted hyaluronan/gelatin) as an anti-adhesion barrier [3], *in situ* crosslinkable hyaluronic hydrogel and autocrosslinked hyaluronan-derived gel [4,5], ultraviolet crosslinked gelatin film [6], sodium alginate [7], sodium carboxymethyl cellulose-heparin [8], and collagen and hyaluronic acid [9]. Anti-adhesion barrier products that are currently commercially available include Interceed (Ethicon, OH, USA) which contains oxidized regenerated cellulose, Gore-Tex (W.L. Gore & Associates Inc., AZ, USA) which is made with expanded polytetrafluoroethylene, Seprafilm (Genzyme Biosurgery, MA, USA) which is composed of sodium hyaluronate and sodium carboxymethyl cellulose, and SprayGel (Confluent Surgical Inc., MA, USA) made with polyethylene glycol. However, the use of these products is limited owing to problems such as flow, early decomposition, and aggregation [10].

Herein we report the fabrication of an injectable pullulan hydrogel that overcomes the drawbacks of other anti-adhesion barriers. Pullulan is a polysaccharide that shows superior adhesive qualities when dissolved in water. This property allows a pullulan hydrogel to reside on injured tissue. This hydrogel would not require removal after use as an anti-adhesion barrier because pullulan is degraded naturally *in vivo* [11]. Hydrogel barriers are advantageous because they cover both complicated and microscopic wounds perfectly, can be used in a spray form, have the flexibility to absorb impacts [12], and have biodegradation periods that can be adjusted through variations in crosslinking density and concentration. Finally, hydrogel can protect injured wound from immune system and permeate oxygen and nutrients [13]. Furthermore, spray or syringe hydrogel formulations are easily used in laparoscopic procedures. Hydrogels are also biocompatible owing to their high water content and

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similarity to the native extracellular matrix [14]. So it can also use not only anti-adhesion barrier but also wound dressing and tissue engineering [13,15].

Given these advantages, it is reasonable to develop postoperative adhesion prevention barriers in hydrogel form. To fabricate the pullulan hydrogel, we modified pullulan with tyramine *via* reaction with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and then formed the hydrogel with horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) [16]. HRP and H_2O_2 produced phenol radical in tyramine which crosslink pullulan. Pullulan as hydrogel was mostly fabricated by trisodium trimetaphosphate (TSTP) as a crosslinking agent for starches [17–20]. But, here we introduce a novel approach to form pullulan hydrogels using HRP and H_2O_2 reaction. Because, polysaccharide such as alginic acid, hyaluronic acid could be form to hydrogel using HRP and H_2O_2 [21,22]. The pullulan hydrogel was characterized with attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), nuclear magnetic resonance spectroscopy (NMR), and rheometric analysis. A cell experiment was conducted to determine whether the surface properties of the hydrogel affect cell viability, and the anti-adhesion potency of the pullulan hydrogel was evaluated with animal models of abdominal defects and cecum adhesion.

2. Materials and methods

2.1. Materials

Pullulan (molecular weight, 100,000 g/mol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Tokyo Chemical Industry (Japan). 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO, free radical), sodium bromide (NaBr), and sodium hypochlorite (NaOCl) solution were purchased from Sigma-Aldrich (USA). *N*-Hydroxysuccinimide (NHS) was obtained from Wako (Japan). Ethanol and hydrogen peroxide (H_2O_2, 30%) were obtained from Daejung Chemicals & Metals (Korea). Horseradish peroxidase (HRP, RZ 1.0, specific activity expressed in terms of pyrogallol units; 113 U/mg, One pyrogallol unit will form 1.0 mg purpurogallin from pyrogallol in 20 s at pH 6.0. at 20 °C) was purchased from Amresco (USA). Purified water was obtained using a water purification system (Pure power I$^+$; Human Corporation, Korea).

2.2. Immobilization of carboxyl groups on pullulan

Pullulan was modified with TEMPO reagent to introduce carboxyl groups as crosslinking sites (Fig. 1). Pullulan, TEMPO, and NaBr were dissolved in 5 °C deionized water at concentrations of 0.800, 0.016, and 0.080% (w/v), respectively. After dissolution of the pullulan mixture, 15% sodium hypochlorite solution was added to the solution at 6% (v/v) while maintaining a pH of 9.5–10 (5 °C, 2 h). Then, 0.5 M NaOH was gradually added while maintaining the same pH (5 °C, 2 h). Methanol was poured into the mixture to terminate the reaction. Finally, the pullulan solution modified with carboxyl groups was neutralized by adding 4 M HCl, and precipitation was completed *via* three rinses with 90% (v/v) acetone solution. The modified pullulan was dried under vacuum (25 °C, 24 h) to remove residual solvents.

2.3. Incorporation of tyramine in carboxylated pullulan with EDC and NHS

The carboxylated pullulan was activated by using EDC with NHS as a proton exchanger to immobilize tyramine on pullulan (Fig. 2) [23,24]. The carboxylated pullulan was dissolved in deionized water at 3% (w/v) for 3 h. Then EDC, NHS, and tyramine were added the carboxylated pullulan solution at 3.06%, 2.56%, and 2.84%, respectively. The mixture was stirred gently for 24 h (25 °C). Thereafter, the mixture solution was dialyzed against the following in sequence: 100 mM NaCl solution for 1 day, 25% (v/v) ethanol solution for 1 day, and deionized water for 1 day. The dialyzed polymer solution was lyophilized for 3 days and kept in a freezer.

2.4. Preparation of pullulan hydrogel

The lyophilized pullulan containing phenyl and hydroxyl groups was dissolved in phosphate-buffered saline solution at 10, 20, 30, and 40 wt%. Then, HRP (226–904 U/200 μl) and H_2O_2 (10–50 μl/10 ml) were added to the modified pullulan solution for gelation (30–60 s). Crosslinking reaction of tyramine modified pullulan was occurred between oxygen radical and ortho-carbon radical or between ortho-carbon radical and ortho-carbon radical by enzymatic oxidative coupling reaction (Fig. 3).

2.5. Characterization of pullulan hydrogel

To confirm the chemical structures of pullulan modified by the TEMPO and EDC-NHS reactions, we used an ATR-FTIR spectrometer (300E; Jasco, Japan) with a Ge ATR crystal (MIRacle, PIKE Technologies, WI, USA). We also used ^{13}C and ^{1}H NMR spectrometers (Advance III 400, 400 MHz; Bruker BioSpin, Germany) for the characterization. To track the molecular weight of pullulan derivatives during pullulan modification, we used a gel permeation chromatography system (Breeze System, with a Waters 2414 Refractive Index Detector; Waters Ultrahydrogel Linear, 120, 250, and 500 column; Pullulan Standard 6100, 9600, 10700, 21100, 47100; Waters, USA) to measure original pullulan, pullulan after the TEMPO reaction, and pullulan after the EDC-NHS reaction at 30 °C. Sodium nitrate ($NaNO_3$, 0.02 N) was used as the eluting solvent (flow rate: 0.8 ml/min). The effects of the concentration of polymer solution, H_2O_2 (crosslinking agent), and HRP (initiator) and rate of tyramine substitution on the storage modulus (G′) of the pullulan hydrogel were investigated by using a rheometer (HAAKE MARS II; Thermo Scientific, USA).

2.6. Cell viability

The reagents used in the cell viability experiments were purchased from Welgene Inc. (Korea). Normal human epidermal fibroblasts were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin G-streptomycin and incubated at 37 °C under 5% CO_2 conditions. The culture medium was exchanged twice a week. The sample was prepared with various amounts of H_2O_2 and sterilized with ultraviolet rays. Cells were seeded on 30 μl of pullulan hydrogel in 24-well plates at 2×10^4 cells/well and allowed to adhere for 4 h at 37 °C in an incubator. The viability of cells was determined with a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay for 4 h on days 1, 3, 5, and 7 after seeding. The culture medium was removed each time, and a 1-ml solution consisting of a 1:9 volume ratio of MTT and DMEM was added to the seeded cell samples. Then, the samples were incubated at 37 °C for 4 h. Incubated solution (200 μl) was moved to 96-well plates. A microplate reader (Opsys MR, DYNEX Tech. Inc., Korea) was used to detect the optical density of formazan at 540 nm.

2.7. Animal tests

Outbred male Sprague-Dawley rats (230–280 g, 8 weeks old) were used as the experimental model. The animal experiments were reviewed and approved by the institutional animal care and use ethics committee of the Kyungpook National University School