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Preparation of gelatin hydrogels incorporating low-molecular-weight heparin for anti-fibrotic therapy

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ABSTRACT

The objective of this study is to design biodegradable hydrogels for the controlled release of low-molecular-weight heparin (LMWH) and evaluate the biological activity. Gelatin was cationized by chemically introducing ethylene diamine into the carboxyl groups in different conditions to obtain cationized gelatins. The cationized gelatin was mixed with the LMWH in aqueous solution to form the complex. Gelatin, together with the complex of LMWH and cationized gelatin, was dehydrothermally cross-linked for different time periods to prepare the gelatin hydrogel-incorporating complex. The hydrogel-incorporating complex was neither degraded in phosphate-buffered saline solution (PBS) at 37 °C nor did it release the LMWH complex. When placed in PBS containing collagenase, the hydrogel was enzymatically degraded to release the LMWH complex. The time profile of hydrogel degradation and the LMWH release depended on the condition of hydrogel cross-linking. The longer the cross-linking time period, the slower the hydrogel degradation and the subsequent LMWH release. The half-life period of LMWH release was in good correspondence with that of hydrogel degradation. It is possible that the LMWH was released as the result of hydrogel degradation. When applied to the mouse model of abdominal membrane fibrosis, the hydrogel system of LMWH release showed a promising anti-fibrotic effect.

1. Introduction

The therapeutic effects of many drugs with different physicochemical and biological properties have been investigated. However, drugs often show side-effects. The use of different drug delivery systems (DDS) has been explored as a practical technology that can modify the biodistribution and the consequent therapeutic effects of drugs. Among the DDS technologies, a variety of materials for the controlled release of drugs have been investigated [1–9]. However, after drug release, the release carrier material sometimes remains, even if these materials are biodegradable. The remaining materials often cause inflammatory reactions and therapeutically unacceptable responses. Therefore, in practice it is necessary to develop a carrier material for drug release systems which does not induce inflammatory reactions. It is well recognized that, compared with hydrophobic polymer materials, hydrophilic materials such as hydrogels show fewer inflammation responses [10,11].

Gelatin is a biodegradable material and has been extensively used for food, drug ingredients, and medical purposes. The biosafety of gelatin has been proven through its long practical applications. Gelatin has various side chains which can be chemically modified with ease. Dehydrothermal or chemical treatment enables gelatin to intermolecularly cross-link to obtain the hydrogel. Gelatin hydrogel can be enzymatically degraded and the degradability can be changed by altering the cross-linking condition. The time period of hydrogel degradation ranged from a few days to several months [12]. We have demonstrated that gelatin hydrogels could release plasmid DNA and proteins with biological activity and enhance their biological activities [13–23].

Heparin is a negatively charged glycosaminoglycan, which is composed of repeated disaccharide units of alternating glucosamine and glucuronic residues heterogeneously modified by carboxyl groups and N- or O-linked sulfate. It has been clinically used as an anticoagulant agent. In addition, other biological effects have been reported. For example, heparin enables cells to stimulate the production of hepatocyte growth factor (HGF) [24]. The anti-fibrotic effect of heparin is experimentally confirmed with a mouse model of CCl_4-induced hepatitis and unilateral ureteral obstruction (UUO) kidney fibrosis [25,26]. Heparin has a side-effect of bleeding acceleration [27]. It is reported that compared with normal heparin, low-molecular-weight heparin (LMWH) has the nature to induce less bleeding [28]. The potential to induce the HGF production is similar to that of normal heparin [29]. Based on these findings, the LMWH was chosen as an anti-fibrotic drug in this study.

The objective of this study is to design a gelatin hydrogel system for the controlled release of LMWH. Cationized gelatin was prepared to form the water-soluble complex of LMWH. The complex was mixed with gelatin, followed by dehydrothermal

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cross-linking to prepare gelatin hydrogels incorporating the complex of LMWH and cationized gelatin. The profile of LMWH release from the hydrogel and the hydrogel degradation was examined while the biological activity of hydrogels was evaluated for a mouse model of peritoneal fibrosis.

2. Materials and methods

2.1. Materials

Gelatin with an isoelectric point (pI) of 5.0 (Mw = 100,000), prepared via an alkaline process of bovine bone (pI5 gelatin) or with a pI of 9.0 (Mw = 100,000), prepared via an acid process of pig skin (pI9 gelatin) and collagenase L, were kindly supplied from Nitta Gelatin Co., Osaka, Japan. LMWH (Mw = 5000, 130 IU mg^{-1}) was kindly supplied from Fuso Pharmaceutical Industries Ltd. Ethylene diamine (EDA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 2,4,6-trinitrobenzene sulfonic acid (TNBS), β-alanine, 1,9-dimethylmethylene blue, sodium dodecyl sulfate (SDS), methanol, formic acid, sodium formic acid, and Cell Count Reagent SF were purchased from Nacalai Tesque, Kyoto, Japan. Testzym® heparin S was purchased from Yashima pure chemicals Co. Ltd. Mild form and chlorhexidine gluconate were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. They were all of reagent grade and used without further purification.

2.2. Preparation of cationized gelatin with different extents of cationization

The carboxyl groups of gelatin were chemically converted by introducing EDA for cationization under different reaction conditions (Table 1). Briefly, various amounts of EDA were added into 25 ml of 0.1 M phosphate-buffered solution (PB, pH = 5.0) containing 1 g of pI9 gelatin. The pH of the solution was adjusted to 5.0 by adding 6 M HCl aqueous solution, and PB was added into the solution to give the final volume of 50 ml. Then, EDC was added into the solution, followed by agitation at 37 °C for 4 h and dialysis against double-distilled water (DDW) for 3 days at room temperature. The dialyzed solution was freeze-dried to obtain cationized gelatins. Cationized extent of cationized gelatin was determined by the conventional TNBS method [30]. Briefly, pI9 gelatin and cationized gelatins were dissolved into 0.1 M phosphate-buffered saline solution (PBS, pH = 7.4) at 1 mg ml^{-1}. Then, 4 wt.% of sodium hydrogen carbonate aqueous solution (200 μl) and 0.1 wt.% of TNBS aqueous solution (200 μl) were added into the gelatin solution (100 μl), and then the mixed solution was allowed to react for 2 h at 37 °C. After reaction, 10 wt.% of SDS aqueous solution (200 μl) and 1 N HCl (100 μl) were added into the mixed solution. The percentage of EDA introduced, the cationization extent was measured from the decrement of amino groups in gelatin by the conventional TNBS method. A calibration curve was prepared with the determined amounts of β-alanine. The absorbance of solutions at 415 nm was determined by the VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The percentage introduced was determined based on the calibration curve and the absorbance of samples.

2.3. Dynamic light scattering and zeta potential measurements of cationized gelatin complexes with or without LMWH

To evaluate the apparent molecular size of cationized gelatins and the LMWH complexes, dynamic light scattering (DLS) measurement was carried out on a DLS-DPA-60HD (Otsuka Electronic Co. Ltd., Osaka, Japan) equipped with a He–Ne laser at a detection angle of 90° at 37 °C. Each sample was dissolved in DDW to give the concentration of 1.0 mg ml^{-1}. The measurement of electrophoretic light scattering (ELS) (ELS-7000, Otsuka Electronic Co. Ltd., Osaka, Japan) was performed at room temperature and with an electric field strength of 100 V cm^{-1}. The complexes were dissolved in 10 mM PBS for the measurement. The experiment was done three times independently for every sample unless otherwise mentioned.

2.4. Cytotoxicity evaluation of cationized gelatin

To evaluate the cytotoxicity of cationized gelatin, an in vitro bioassay with L929 fibroblasts was carried out. Each well of 96-well multiwell culture plate (Corning Inc., NY) was coated with cationized gelatin solution in PBS. Briefly, the cationized gelatin solution (1 mg ml^{-1}) was placed into each well. After 30 min, the solution was removed, and the well was washed by PBS (100 μl) three times. L929 cells were seeded into each well of 96-well cell culture plate with pI9 gelatin or cationied gelatin coating at a density of 10,000 cells/100 μl per well. They were cultivated in the Dulbecco's modified Eagle medium (DMEM) containing 10 vol.% fetal bovine serum (FBS) and 1.0 vol.% penicillin/streptomycin for 6, 24, and 48 h at 37 °C in 5% CO_2–95% air atmosphere. The medium was exchanged to 100 μl of fresh medium containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) of Cell Count Reagent SF and incubated further for 2 h. The absorbance of medium was measured at 450 nm. The percentage of cell viability was expressed as 100% for cells cultured with pI9 gelatin. The experiment was done five times independently for each sample.

2.5. Preparation of gelatin hydrogels incorporating LMWH and cationized gelatin complexes

LMWH was mixed with the cationized gelatin in DDW (1 ml) at 37 °C to prepare the complex of LMWH and cationized gelatin (Ta-

Table 1
Preparation and characterization of cationized gelatins and the complexation with LMWH.

Code	Gelatin concentration (g/ml)	EDA concentration (ml)	EDA used molar ratio[a]	Percentage of EDA introduced[b]	Amount of cationized gelatin used for complexation[c] (mg)
E–0.5	1	0.031	0.5	4.6 ± 3.8	37.5
E–1.0	1	0.063	1.0	16.7 ± 1.8	35.0
E–2.0	1	0.125	2.0	19.8 ± 5.4	35.0
E–3.0	1	0.188	3.0	27.0 ± 2.8	25.0
E–5.0	1	0.314	5.0	31.1 ± 3.9	22.5
E–7.0	1	0.439	7.0	36.0 ± 1.2	22.5
E–10	1	0.627	10	38.3 ± 3.9	17.5
E–50	1	3.14	50	56.5 ± 4.5	7.5

[a] The molar ratio of EDA added to the carboxyl groups of gelatin.
[b] The molar percentage of EDA introduced to the carboxyl groups of gelatin.
[c] The amount for 2.5 mg of LMWH.