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 were dehydrothermally crosslinked for different time periods to prepare gelatin hydrogels incorporating complex. The hydrogel incorporating complex was neither degraded in phosphate-buffered saline solution (PBS) at 37 °C nor 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 crosslinking. The longer the crosslinking 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 for anti-fibrotic effect.

Keywords: Gelatin hydrogel, Controlled release, LMWH, Anti-fibrotic therapy

Introduction

There have been investigated the therapeutic effect of many drugs with different physicochemical and biological properties. However, the drugs often show the side effects. As one trial to dissolve the problems, drug delivery system (DDS) is one of practically possible technologies that can modify the biodistribution and the consequent therapeutic effects. Among the DDS technologies, a variety of materials for the controlled release of drugs have been investigated [1-9]. However, after the drugs release, the material of release carrier sometimes remains even if they are biodegradable. The materials' remaining often causes inflammatory reactions and therapeutically unacceptable responses. Therefore, it is practically necessary to develop the carrier material of drug release which does not induce inflammatory reactions. It is well recognized that comparing with hydrophobic polymer materials, hydrophilic materials like hydrogels show less inflammation responses [10, 11].

Gelatin is a biodegradable material and has been extensively used for food, drug ingredients, and medical purposes. The biosafety has been proven through their long practical applications. Gelatin has various side chains which can be chemically modified with ease. Dehydrothermal or chemical treatment enables gelatin to intermolecularly crosslink to obtain the hydrogel. Gelatin hydrogel can be enzymatically degraded and the degradability can be changed by altering the crosslinking condition. The time period of hydrogels 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 functions 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 CCl₄-induced hepatitis and unilateral ureteral obstruction (UUO) kidney fibrosis [25, 26]. Heparin has a side effect of bleeding acceleration [27]. It is reported that comparing 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 finding, 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 LMWH. Cationized gelatin was prepared to form the water-soluble complex of LMWH. The complex was mixed with gelatin, followed by the dehydrothermal crosslinking 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.

MATERIALS AND METHODS

Materials

Gelatin with an isoelectric point (pI) of 5.0 (Mw=100,000), prepared through an alkaline process of bovine bone (pI5 gelatin) or with a pI of 9.0 (Mw=100,000), prepared through an acid process of pig skin (pI9 gelatin) and collagenase L were kindly supplied from Nitta Gelatin Co., Osaka, Japan. LMWH (Mw=5,000, 130 IU/mg) 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.

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 solution pH 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 the agitation at 37 °C for 4 hr 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. 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 hr at 37 °C. After reaction, 10 wt% of SDS aqueous solution (200 μ l) and 1N HCl (100 μ l) 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 percent introduced was determined based on the calibration curve and the absorbance of samples.

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. The measurement of electrophoretic light scattering (ELS) (ELS-7000, Otsuka Electronic Co. Ltd., Osaka, Japan) was performed at room temperature and electric field strength of 100 V/cm. The complexes were dissolved in 10 mM PBS for the measurement. The experiment was done three times independently for every sample unless otherwise mentioned.

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 cationied gelatin solution in PBS. Briefly, the cationized gelatin solution (1 mg/ml) was placed into each well. After 30 min, the solution was removed, and the well was washed by PBS (100 μ l) 3 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 hr at 37 °C in 5% CO2-95% 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 hr. 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 5 times independently for each sample.

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 (Table 1). Next, aqueous solution of pI5 gelatin (20 wt%, 1 ml) was added to the complexes solution at 37 °C. The mixed solution was poured into a polytetrafluoroethylene mold and frozen in liquid nitrogen, followed by freeze-drying. After that, the hydrogels were placed into the dry oven (AURORA DN-305, Sato Vacuum Inc., Japan) and dehydrothermally crosslinked for 12, 24, 48, 72, and 96 hr at 160 °C.

Release test of LMWH from gelatin hydrogels incorporating LMWH and cationized gelatin complexes

Hydrogels of 5 to 7 mg in dry weight were placed in PBS (500 μ l) at 37 °C. The PBS supernatant was exchanged 1, 2, 4, 8, 24, 48, and 96 hr later and subsequently fresh PBS was added. The release test was performed in PBS for the initial 24 hr and after that newly performed in PBS containing collagenase (1 mg/ml). The amount of LMWH in each solution was determined by the conventional colorimetric method [31]. Briefly, 1,9-dimethylmethylene blue (DMB, 1.6 mg) was dissolved in methanol (0.5 ml). Formic acid (0.2 g) and sodium formic acid (0.2 ml) were added to DDW to give the final volume of 100 ml. Sample solutions (25 μ l) were mixture with DMB solution (225 μ l). A

calibration curve was prepared with the determined amounts of LMWH. The absorbance of solutions at 525 nm was determined by the microplate reader. The percent introduced was determined based on the calibration curve and the absorbance of samples.

Degradation evaluation of hydrogels incorporating LMWH and cationized gelatin complex

Hydrogels of 5 to 7 mg in dry weight were placed in PBS (500 μ l) at 37 °C. The PBS superratant was exchanged 1, 2, 4, 8, and 24 hr later and subsequently fresh PBS was added. After that, the hydrogels were newly performed in PBS containing collagenase (1 mg/ml). PBS containing collagenase superratant was exchanged every 3 hr and fresh one was added. The amount of gelatin in each solution was determined by the colorimetric method [18]. The absorbance of solutions at 280 nm was determined by the microplate reader. A calibration curve was prepared with the determined amounts of pI5 gelatin. The percent introduce was determined based on the calibration curve and the absorbance of samples.

Evaluation of anti-fibrotic effects

C57B/L6 mice were purchased from Shimizu Laboratory Supplies Co. Kyoto, Japan. All the animal experimentation was conducted according to the guidance of the Institute for Frontier Medical Sciences, Kyoto University. Peritoneal fibrosis model of mice was prepared [32]. Briefly, mice, 9- to 10-week old female, weighing 20 g were intraperitoneally administered with 0.1 vol% chlorhexidine gluconate dissolved in 15 vol% ethanol solution (15 μ g/g body weight) every 3 days at 7 times. Gelatin hydrogels of 2.0 to 2.2 mg incorporating the LMWH and E-7.0 cationized gelatin complex were implanted 21 days later in right side of mice peritoneal. The left side of mice peritoneal was not treated. The hydrogels incorporating complex were applied on the peritoneal wall and sutured for the fixation 3, 7, 10, 14, and 21 days after hydrogel application. The hydrogel incorporating complex was crosslinked for 12 and 24 hr. As controls, the complex-free hydrogel was applied and mice were not treated. The abdominal wall containing the hydrogel was taken and fixed in 4 vol%

paraformaldehyde solution. They were embedded in paraffin and histological sections with 4 μ m thickness were prepared by the rotary microtome (RM2125RT, Leica Microsystems Inc., Osaka, Japan). The sections were stained with hematoxylin and eosin staining and Masson trichrome without nucleus staining and viewed to evaluate the thickness of fibrotic walls on microscope (PROVIS AX80, Olympus Co., Japan). Histological sections randomly selected (2 sections for each experimental group) were observed and the average thickness was calculated.

Evaluation of LMWH activity of hydrogels incorporating LMWH and cationized gelatin complexes

Gelatin hydrogels incorporating LMWH and cationized gelatin complexes were completely dissolved in PBS containing 1 mg/ml of collagenase at 37 °C. The hydrogels were crosslinked for 12 and 24 hr at 160 °C while non-crosslinked hydrogels were used as a control. The LMWH activity was evaluated by using Testzym®heparin S (Sekisui medical Co. Ltd., Osaka Japan). Briefly, hydrogels were dissolved by collagenase solution and the resulting solution (10 μ l) was mixed with a plasma solution (10 μ l), 1.0 IU/ml of antithrombin III solution (10 μ l) and a 50 mmol/l of 2-Amino-2-methyl-1,3-propanediol buffer solution (pH=8.4, 70 μ l) for 6 min at 37 °C. Factor Xa solution (50 μ l, 7.1 nkt/ml) was added into the mixed solution for 30 sec at 37 °C. N-benzoyl-L-isoleucyl-L-glutamyl (γ -OR) - glycyl-L-arginyl-p-nitroanilide hydrochloride (100 μ l) was added into the mixed solution for 3 min at 37 °C. Immediately after that, 50 vol% of acetic acid was added into this mixture to stop the reaction. A calibration curve was prepared with the determined amounts of LMWH. The absorbance of solutions at 405 nm was determined by the microplate reader.

Statistical analysis

All the results were expressed as the mean \pm standard deviation (SD). Significant analysis was done based on the one-way ANOVA, and the difference was considered to be significant at p < 0.05.

RESULTS

Characterization of cationized gelatin and LMWH complexes

Table 1 summarizes the characterization of cationized gelatin and the complexes with LMWH. The cationization extent of gelatin increased with an increase in the concentration of EDA added. Figure 1 shows the apparent molecular size and zeta potential of cationized gelatin with different cationization extents and the LMWH complexes. The molecular size of cationized gelatins and the LMWH complex tended to decrease when the cationization extent of gelatin with cationization extent of 27.0 % or lower. However, at the higher extents, the complex size became smaller. The zeta potential of cationized gelatin was higher than that of the corresponding complexes and tended to increase with increasing cationization extent of gelatin. At the cationization extent of 56.5 %, the zeta potential of complexes was negatively charged.

Figure 2 shows the cytotoxicity of cationized gelatins. Cationized gelatins at lower extents of cationization showed no cytotoxicity whereas the E-10 and E-50 cationized gelatin did cytotoxicity.

In vitro time profiles of LMWH release from gelatin hydrogels incorporating LMWH and cationized gelatin complexes

Figure 3 shows in vitro time profiles of LMWH release from gelatin hydrogels incorporating LMWH and cationized gelatin complexes in PBS with or without collagenase. With increasing the cationization extent of gelatin, the initial burst release of LMWH tended to be suppressed. When examined in PBS, the gelatin hydrogels incorporating LMWH and cationized gelatin complexes showed similar release profile, irrespective of the time period of hydrogel crosslinking. On the contrary, in PBS containing collagenase, the time profile of LMWH release depended on the crosslinking time. The LMWH release became slower as the time of hydrogel crosslinking became longer.

In vitro time profiles of hydrogel degradation

Figure 4A shows in vitro time profiles of hydrogel degradation in PBS with or without collagenase. With increasing the crosslinking extent of the hydrogels, the initial burst degradation of hydrogels tended to be suppressed. When examined in PBS, the gelatin hydrogels incorporating LMWH and cationized gelatin complexes showed similar degradation profile, irrespective of the time period of hydrogel crosslinking. On the contrary, in PBS containing collagenase, the time profile of hydrogel degradation depended on the crosslinking time. The hydrogel degradation became slower as the time of hydrogel crosslinking became longer.

Figure 4B shows the relationship of half-life period between the LMWH release and hydrogel degradation. The half-life period was determined from the time profiles of LMWH release and hydrogel degradation in the test with collagenase. A good correlation in the half-life time between the LMWH release and hydrogel was observed.

Anti-fibrotic effects of gelatin hydrogels incorporating LMWH and cationized gelatin

Figure 5 shows histological sections of peritoneal fibrosis tissues before (A) and 21 days after (B) application with hydrogel incorporating LMWH and cationized gelatin which is prepared through dehydrothermal crosslinking 12 hr. Apparently, the application of hydrogels incorporating LMWH and cationized gelatin complex decreased the thickness of fibrotic peritoneal walls.

Figure 6 shows the anti-fibrotic effects of gelatin hydrogels implanted for a mouse model of abdominal fibrosis (A). Significant anti-fibrotic effect was observed for the hydrogels incorporating complex 10, 14, and 21 days after the application, irrespective of the hydrogel crosslinking time. On the contrary, the complex-free hydrogels did not show any biological activities, which is similar to that of non-treated control group. On the other hand, no significant anti-fibrotic effect of non-treated left side was observed (B). The peritoneal wall thickness at the site applied with the complex-free hydrogel was similar to that of non- treatment group.

Figure 7 shows the LMWH activity collected from gelatin hydrogels incorporating LMWH and cationized gelatin complexes. The LMWH activity remaining was detected even though it was dehydrothermally crosslinked.

Discussion

This study demonstrates that the biodegradable gelatin hydrogel could release the LMWH of biological activity in different release profiles when the LMWH complex with cationized gelatin was incorporated into the hydrogel. Gelatin and the derivatives have been used to prepare the hydrogel of carrier material for the controlled release of various drugs, proteins, and genes, because of the biodegradability and biocompatibility. When the LMWH was directly into the hydrogels prepared by the dehydrothermal crosslinking of pI5 or pI9 gelatin, an initial burst in LMWH release (about 80 %) was observed (data not shown). This is because the LMWH of small molecule did not interact with the gelatin molecules and the consequent release by the simple diffusion. Therefore, the LMWH of negative charge was mixed with the cationized gelatin to form nano-sized complexes. The complex of positive charge was immobilized into the hydrogel of pI5 gelatin with negative charge. The initial burst of LMWH release was effectively suppressed (Figure 3A). It is possible that the complex with positive charge (Figure 1B) interacted with pI5 gelatin, resulting in the suppressed initial burst. The release test in collagenase solution revealed that the LMWH release became slower as the time period of hydrogel crosslinking (Figure 3B). At the cationization extent of 56.5 %, the zeta potential of complexes was negatively charged. This is because the complex surface had an excessive positive charge which can be firmly interacted with the LMWH. The strong interaction would make the surface charge negative. This complex system could not be interacted with pI5 gelatin. Therefore, the initial burst release of LMWH from gelatin hydrogels incorporating LMWH and E-50 cationized gelatin was larger than that of E-7.0 and E-10 cationized gelatin (Figure 3A).

The time period of LMWH release well corresponded with that of hydrogel degradation (Figure 4B). The extent of hydrogel crosslinking would increase with an increase in the crosslinking time. It is highly conceivable that the hydrogel was degraded enzymatically to generate water-soluble gelatin fragment, resulting in the release of LMWH in the complexed state. The complex had positive charged although the charge was lower than that of cationized gelatin. This positive charge is necessary to electrostatistically interact with the pI5 gelatin of negative charge, but sometimes

causes cytotoxicity. Cationized materials show the cytotoxicity higher than anionized ones [33]. It is apparent that cationized gelatin with higher extents of cationization showed cytotoxicity (Figure 2). Taken together, the E-7.0 cationized gelatin was selected to form the complexes with LMWH.

Gelatin hydrogels have been explored for the release carrier of various drugs [13-23]. We have demonstrated that various growth factors of biological activity, such as basic fibroblast growth factor [34], transforming growth factor- β 1[35], bone morphogenic protein-2 [36], and hepatocyte growth factor [37] etc., could be released to enhance their induction potentials of tissue regeneration. In the gelatin hydrogel system, the growth factor is immobilized into the hydrogel matrix through the physicochemical interactions between the factor and gelatin molecules. The factor immobilized can be released from the hydrogel only when the hydrogel is degraded enzymatically to generate water-soluble gelatin fragments. Therefore, the time period of factor release can be regulated only by that of hydrogel degradation. In the in vitro release test with collagenase-free PBS, the factor immobilized is not release although the small amount of initial release is observed for the factor without interaction with gelatin matrix. However, when the release test is performed in collagenase solution, the hydrogel is enzymatically degraded with time and consequently the factor immobilized is released from the hydrogel. The present LMWH release is based on this mechanism of carrier matrix degradation. The LMWH-complexes are immobilized in the gelatin hydrogel matrix. When degraded by collagenase, the hydrogel can release the LMWH-complexes, as shown in Figure 3B. The time profile of LMWH is regulated by that of hydrogel degradation.

The hydrogels incorporating LMWH and cationized gelatin showed significant anti-fibrotic effects (Figures 5, and 6). It is reported that the LMWH promotes the production of HGF which has an anti-fibrotic activity [25, 26]. Several researches have been reported on the HGF-based anti-fibrotic activity [38-40]. There are some mechanisms about that. HGF is an ability to induce the Matrix Metalloproteinase (MMP) -1 production, which can digest the fibrosis of collagen, resulting in

reduced fibrosis [41]. It is well known that transforming growth factor (TGF) - β 1 has been implicated as a major inducer of fibrosis in many tissues [42]. On the other hand, HGF has an ability to suppress the TGF- β 1 production [43]. It is possible that HGF suppress the TGF- β 1 production, resulting in the reduced progression of fibrosis. We can say with certainty that the local release of LMWH functioned the surrounding cells to induce the HGF production, resulting in the HGF-induced anti-fibrotic effect. The peritoneal wall thickness at the site applied with the complex-free hydrogel and without any treatments had no significant effects. This indicates that the hydrogels incorporating E-7.0 cationized gelatin are of non- or low-inflammation induction.

In conclusion, the biodegradable hydrogel of gelatin has a potential to enable LMWH to release for enhanced in vivo biological activity, resulting in promoted therapeutic efficacy of anti-fibrosis. The release technology can be applied to various types of low-molecular-weight drugs. When formulated into nano or micro-particles, the hydrogel can be injected.

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FIGURE CAPTIONS

Figure 1. Apparent molecular sizes (A) and zeta potentials (B) of cationized gelatins
(•) and the LMWH complexes (•) as a function of the cationization extent.

Figure 2. Cytotoxicity of cationized gelatins with different cationization extents. L929 cells were cultured in the presence or absence of cationized gelatins for 6 (\Box), 24 (\blacksquare), 48 hr (\blacksquare). The percentage of cell viability was expressed as 100 % for cells cultured with pI9 gelatin. *, P<0.05; significant against the viability of cells cultured with pI9 gelatin at the corresponding time period.

Figure 3. In vitro release profiles of LMWH from gelatin hydrogels incorporating complexes of LMWH and cationized gelatin (A). The complexes were prepared for LMWH with E-0.5 (•), E-1.0 (\blacktriangle), E-2.0 (•), E-3.0 (\diamondsuit), E-5.0 (\circ), E-7.0 (\triangle), E-10 (\Box), and E-50 cationized gelatin (\diamondsuit) in PBS at 37 °C. The hydrogels incorporating complex were dehydrothermally crosslinked at 160 °C for 24 hr. (B) The hydrogels incorporating complex of LMWH and E-7.0 cationized gelatin were dehydrothermally crosslinked at 160 °C for 12 (\circ), 24 (\bullet), 48 (\triangle), 72 (\bigstar), and 96 hr (\Box). The release test was performed in PBS for the initial 24 hr and there after in collagenase solution in PBS at 37 °C. **Figure 4.** (A) In vitro degradation profiles of gelatin hydrogels incorporating complex of LMWH and E-7.0 cationized gelatin. The hydrogels were dehydrothermally crosslinked at 160 °C for 12 (\circ), 24 (\bullet), 48 (\triangle), 72 (\bigstar), and 96 hr (\Box). The degradation test was performed in PBS for the initial 24 hr and thereafter in collagenase solution in PBS at 37 °C. (B) The relationship of half-life period between the LMWH release and hydrogel degradation. The half-life period was determined from the time profiles of LMWH release and hydrogel degradation in the test with collagenase.

Figure 5. Histological sections (H&E and Masson Trichrome staining) of peritoneal fibrosis tissues (A and B) and 21 days after application with hydrogel incorporating LMWH and cationized gelatin which is prepared through dehydrothermal crosslinking 24 hr (C) and without any treatment (D).

Figure 6. Anti-fibrotic effects of gelatin hydrogels incorporating complex of LMWH and E-7.0 cationized gelatin. The peritoneal wall thickness was measured at the site applied with the hydrogels incorporating complex (■, ■) and the complex-free hydrogel
(■) or without any treatments (□). The hydrogels were dehydrothermally crosslinked at 160 °C for 12 (■) and 24 hr (■).

*, P<0.05; significant against the tissue thickness of non-crosslinked hydrogels.
*, P<0.05; significant against tissue thickness of complex-free hydrogels.

Figure 7. Biological activity of LMWH collected from gelatin hydrogels incorporating the complex of LMWH and E-7.0 cationized gelatin. *, P<0.05; significant against the LMWH activity of non-crosslinked hydrogels.



Figure 1. Saito et al



Figure 2. Saito et al



Figure 3. Saito et al



Figure 4. Saito et al



Figure 5. Saito et al



Figure 6. Saito et al



Figure 7. Saito et al

Code	Gelatin concentration	EDA concentration	EDA used	Percrntage	Amount of cationized gelatin
	(g/ml)	(ml)	molar ratio ^a	of EDA introduced ^b	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

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

^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