Release properties of atelocollagen-gelatin complexes as carriers for local administration of fluvastatin

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The aim of this study was to investigate properties of atelocollagen/gelatin complexes (AC/Gel) and their characteristics of sustained statin release, to assess the utility of AC/Gel. AC/Gel were prepared by changing the mixing ratio of AC (0 to 40% of AC). Analysis of spectra of fluvastatin (Flu), gelatin (Gel), and Flu with Gel complex using a Fourier transform-infrared spectrometer indicates that Flu was bound to Gel through a bond involving the carboxyl and amino groups. Evaluation of characteristics of sustained release of Flu from the AC/Gel using an ultraviolet-visible spectrophotometer showed that the release rate of Flu decreased with increasing the AC content. The histological evaluation using of Sprague-Dawley rats suggest that, unlike the pure Gel sponge, the AC/Gel was not absorbed in an early stage. Therefore, the present study showed that sustained Flu release can be controlled by using an AC/Gel, suggesting the utility of this composite material.

Keywords: Fluvastatin, Atelocollagen, Gelatin, Controlled release, Regeneration

INTRODUCTION

Statins, which are competitive inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme, are widely used to suppress cholesterol synthesis for the treatment of hyperlipidemia. In addition to the cholesterol-lowering effect, statins increase BMP-2 expression, decreasing the activity of osteoclasts and stimulating osteoblast differentiation, promoting bone formation¹⁾. Recently, the promoting effect of statins on bone formation has drawn attention to the field of dentistry and has been extensively studied for possible applications, such as accelerating healing at extraction sockets and implant sites^{2,3)}.

Systemic administration of high-dose statins may increase the risk of side effects, such as rhabdomyolysis and myopathy⁴⁾. Therefore, local administration of statins is being attempted to avoid the risk of side-effects. However, simple direct administration of statins to the affected sites is not effective for osteoinduction because the drug diffuses into the surrounding tissues⁵⁻⁷⁾.

Accordingly, controlled release systems for statins are required for local administration of statins with carriers, such as gelatin, atelocollagen sponge (Teruplug®), α -TCP, β -TCP, HA, and PLGA⁸⁻¹¹⁾. In particular, gelatin is widely used as a drug carrier because of desirable characteristics including (1) proven safety, (2) wide application of this biodegradable material for clinical purposes, (3) different physical properties by chemical modification, and (4) ease of

attaching to drugs¹²⁻¹⁵⁾. Moreover, gelatin derivatives obtained by acid or alkali treatment have different isoelectric points. Such gelatin derivatives are capable of carrying positively or negatively charged substances *via* electrostatic interactions¹⁶⁾.

In our earlier studies, we have investigated fluvastatin sodium, an amphiphilic statin that has both membrane permeability and water solubility¹⁷⁾, which have the properties that are advantageous for local administration. We conjugated fluvastatin sodium to a nontoxic, thermally crosslinked gelatin sponge and showed that the resulting sponge-form conjugates promoted bone formation *in vivo*¹⁸⁻²⁰⁾. However, gelatin is water-soluble, and the thermal crosslinking treatment could prolong dissolution time by only approximately 3 days²⁰⁾. Therefore, the development of carriers capable of more stable drug release is required.

In the present study, we focused on atelocollagen as a statin carrier. Collagen, which forms gelatin on partial hydrolysis, has lower susceptibility to proteases than gelatin²¹⁾ because of its distinctive triple helix structure. Atelocollagen is a collagen derivative from which collagen components responsible for antigenicity have been removed, providing it a greater potential as a biomaterial²²⁾. Since collagen is easy to aggregate in an aqueous phase because of its conformation, it is more hydrophobic than water-soluble gelatin. Therefore, it appeared impossible to immobilize fluvastatin on atelocollagen by electrostatic interactions. Accordingly, we decided to prepare an atelocollagen/gelatin complex in which gelatin functioned as the fluvastatin carrier, as in our earlier studies, and to make efficient use of atelocollagen for retarding degradation of the carrier.

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Prior to the clinical application of the atelocollagen/gelatin complex as a statin carrier, it is necessary to determine (1) properties of the atelocollagen/gelatin complex (internal structure, statin-binding mechanism, and bio-absorbability) and (2) characteristics of sustained release of the statin from the atelocollagen/gelatin complex. However, no relevant previous reports were available.

Therefore, the present study aimed to investigate properties of atelocollagen/gelatin preparations and their characteristics of sustained statin release, to assess the utility of this class of complex.

MATERIALS AND METHODS

Atelocollagen/gelatin complexes preparation

Fluvastatin sodium salt (Flu; Lot WEG3258, Toronto Research Chemicals, Ontario, Canada), alkaline gelatin derived from porcine (Gel; isoelectric point of 7.0–9.0, Nitta Gelatin, Osaka, Japan) and type 1 atelocollagen derived from bovine (AC; Lot No.818025, Koken, Tokyo, Japan) were used in this study.

Three kinds of solutions were prepared as shown below; Solution A: 90 mg of AC was dissolved to 10 mL of final 100 mM phosphoric acid (0.9 AC solution). Solution B: Mixture of 90 mg of Gel and 870 μ M Flu was dissolved to 10 mL of the phosphoric acid (0.9% Gel solution with Flu) according to the early study¹⁸⁾. Solution C: 90 mg of Gel was dissolved to 10 mL of the phosphoric acid (0.9% Gel solution without Flu). The above solutions of A and B as Flu containing group, or of A and C without Flu group, were mixed in a mixing ratio of the solution A, as describing in Table 1. Each solution was mixed thoroughly, continuous stirring for 48 h at 4°C. For preparing group with Flu, solutions of A and B were mixed, named F-AC0, 10, 20, 30, and 40 (The concentration of Flu involved in F-AC0, 10, 20, 30, and 40 were 870, 783, 696, 609, 522 μ M, respectively). For preparing group without Flu, solution of A and C were mixed named AC0, 10, 20, 30, and 40. The mixture was injected in a cylindrical container having an inner diameter of 16 mm and a height of 17 mm, and then was frozen at –20°C for 2 h. Frozen samples were placed in

a freeze dryer (FREEZONE®2.5, LABCONCO, Kansas city, MO, USA) and dried under vacuum at –50°C for 24 h to prepare dry sponges. Finally, atelocollagen/gelatin complex were polymerized by cross-linking the sponge with heat treatment at 125°C for 30 min.

Observation with a scanning electron microscope (SEM)

Fabricated complexes were observed using a scanning electron microscope (SU-6600, Hitachi, Tokyo, Japan). Each complex used for SEM specimen (F-AC0, 10, 20, 30, and 40) was cut in thin sections with a razor blade. Before SEM observation, the cross-sectioned complexes were coated with Au-Pd using a sputter coater (SC500A, Elminet, Tokyo, Japan).

Fourier transform-infrared spectroscopy (FT-IR)

Spectra of Flu, Gel, and F-AC0 were obtained by a Fourier transform-infrared spectrometer (FT-IR430, JASCO, Tokyo, Japan). FT-IR spectra were obtained from discs containing 1 mg sample in approximately 100 mg potassium bromide (KBr). The measuring was performed at a resolution of 1 cm^{-1} for 100 times iterations in the wavelength range from 4,000 to 400 cm^{-1} . The obtained spectra (1,600–1,500 cm^{-1}) were analyzed using a software (KnowItAll Informatics System, Bio-Rad, Hercules, CA, USA).

Release test of fluvastatin from the complexes

The complexes containing Flu (F-AC0 to 40) was used as experimental groups and complexes without Flu (AC0 to 40) were used as control groups (each complex $n=6$). Each complex was filled into 2 mL of phosphate buffered saline (PBS), and then they were stored in a dark box at 37°C. After sampling 60 μ L of each solution eluted from the complex, released Flu from the complexes were measured with absorbance of 303 nm using an ultraviolet-visible spectrophotometer (V-660, JASCO) at 6 h, 24 h, and then every day until 1 week from starting of release test. The dissolution rate was defined as absorbance ratio of eluate in each complex at appropriate period against initial F-AC solution before forming the complex. Ratio was calculated according to the following formula. Dissolution rate (%)=(Absorbance

Table 1 Components of atelocollagen/gelatin complex

AC/Gel complex with Flu	A		AC/Gel complex without Flu	C	
	0.9% AC solution (%)	0.9% Gel solution with Flu (%)		0.9% AC solution (%)	0.9% Gel solution without Flu (%)
F-AC40	40	60	AC40	40	60
F-AC30	30	70	AC30	30	70
F-AC20	20	80	AC20	20	80
F-AC10	10	90	AC10	10	90
F-AC0	0	100	AC0	0	100

of eluate collected in each section/Absorbance of initial F-AC solution of each complex) $\times 100$.

Bioabsorbable experiment

Fifteen-week-old, male, Sprague-Dawley rats ($n=3$) obtained from Sankyo Laboratory (Tokyo, Japan) were used in the experiment. The rats were allowed food and water ad libitum and maintained on a 12 h light/dark cycle (lights on from 8:00 to 20:00) at $23\pm 1^\circ\text{C}$ with $60\pm 10\%$ humidity during the experiment.

All rats, after isoflurane (Wako, Osaka, Japan) inhalation pentobarbital sodium (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan) were anesthetized by intraperitoneal injection of $0.7\text{ }\mu\text{L/g}$ body weight. Thigh was blunt dissection of muscle added after incision that has been shaved. After removing the periosteum to expose the femur, a 1.6 mm round bur was used to create a cylindrical bone defect with 1.6 mm in diameter and depth under constant irrigation with normal saline solution to prevent overheating of the bone edges on either side femoral central portion. The complexes were cut into pieces 1.6 mm in diameter and height to fit the bone defect area.

After hemostasis, AC40 complex without Flu as an experimental group, or AC0 as a control group, was implanted into the defect site, and the site was sutured. All animal experiments in this study were conducted in accordance with the Tokyo Dental College Guidelines for Animal Experimentation (Approval date: 4/1/2015; Approval number: 273002).

The rats were humanely sacrificed with intraperitoneally administered overdoses of pentobarbital at 3, 5, and 7 days after surgery. The removed femurs were immersed in 10% neutral

buffered formalin for 7 days, and paraffin specimens were made after decalcification with 10% EDTA (pH 7.0–7.5) (Wako) for 7 days at room temperature. The specimens were sliced into $3\text{ }\mu\text{m}$ -thick sections and stained with hematoxylin and eosin staining (H-E staining) according to standard protocols. These samples were morphologically observed using a universal photomicroscope (Axiophot 2, Carl Zeiss, Oberkochen, Germany).

Statistical analysis

All data are expressed as the mean $\pm$ standard deviation (SD). The data were analyzed using a statistical analysis software package (GraphPad Prism6, MDF, Tokyo, Japan). Data were first analyzed by one-way ANOVA. When this analysis suggested a significant difference between groups ($p<0.01$), the data were further analyzed by the Tukey *post hoc* multiple comparison tests.

RESULTS

Observation with a scanning electron microscope (SEM)

SEM images of complexes of F-AC0, 10, 20, 30, and 40, shown in Fig. 1 revealed that all preparations had an internal network structure composed of continuous microscopic pores of approximately $200\text{ }\mu\text{m}$.

Fourier transform-infrared spectroscopy (FT-IR)

The results of FT-IR analysis is shown in Fig. 2. Characteristic peaks of Gel and Flu were confirmed, and a mixture of the two, F-AC0, showed changes in peaks. In Fig. 2, the spectra are shown focusing on the $1,600\text{--}1,500\text{ cm}^{-1}$ range, in which marked peak changes are observed. The peak at $1,587\text{ cm}^{-1}$, a carboxyl group-

Fig. 1 SEM images of each complex of F-AC0, 10, 20, 30, and 40. (a)–(e) were setting at magnification $\times 60$. (f)–(j) were setting at magnification $\times 200$. Scale bars show length of $200\text{ }\mu\text{m}$.

Fig. 2 FT-IR analyses ranging from 1,600~1,500 cm^{-1} .

Fig. 3 Dissolution rate of the each complex at 6 h, 24 h, and then every day until 1 week from starting of release test. Error bars represent means $\pm$ standard deviation for $n=6$.

Fig. 4 Dissolution rate of each complex containing Flu. One-way ANOVA Tukey's multiple comparisons tests ($n=6$). Significant differences were observed between each complexes ($p<0.01$). Different alphabetic character (a–e) indicates significant differences.

derived peak that was observed in Flu, was disappeared by mixing Flu and Gel. Peaks at 1,557 and 1,546 cm^{-1} , amino group-derived peaks observed in Gel, were shifted to 1,554 and 1,539 cm^{-1} , respectively.

Release of fluvastatin from the complexes

On the visual observation, F-AC0 began to dissolve immediately after PBS injection in the release test of Flu, and no structure was visible 6 h after initiating the test. Compared with F-AC0, the complex structure persisted for a greater period as the AC ratio increased. Seven days after initiating the test, F-AC30 and 40 retained the complex structure, whereas F-AC10 and 20 lost the structure and retained a visible residue (data not shown).

Dissolution rate (percentages of Flu released) calculated based on absorbance values obtained in the release test of Flu are shown in Figs. 3 and 4. Significant differences were recognized between each complex ($p<0.01$), and the release rate significantly decreased

with increasing AC content. On the control AC0, 10, 20, 30, and 40 groups not containing Flu, no noticeable absorbance at wavelength of 303 nm was observed. With regard to F-AC0, the percentage of elution approached 90% on the first day and gradually increased. With regard to F-AC10 and 20 with low AC content, the dissolution rate were 59 and 52%, respectively, on the first day and gradually increased to 77 and 62% thereafter. With regard to F-AC30 and 40 with high AC content, the dissolution rate gradually increased from 21 and 12% elution at day 1 to 41 and 22% at day 7, respectively.

Bioabsorbable experiment

The complex absorption process in animal experiments is shown in Fig. 5. No abnormal bleeding or inflammation was observed in any animal experiment that was conducted. In animals that received AC0, Gel sponge residues were observed on postoperative day 3. No sponge structure was visible on postoperative

pore size and porosity of sponge-like scaffolds used in regenerative therapy markedly affect cell proliferation, and approximate pore diameters ranging from 200 to 350 μm have been reported to be effective for bone tissue regeneration^{28–30}. Therefore, the atelocollagen/gelatin complexes prepared in this study may be applicable as scaffolds for bone tissue regeneration.

The infrared spectroscopic analysis in this study showed that, in F-AC0 spectra, the peak at 1,587 cm^{-1} derived from the Flu³¹ carboxyl group was absent and the peaks at 1,557 and 1,546 cm^{-1} from the amino group of Gel³² shifted to 1,554 and 1,539 cm^{-1} . This result indicates that Flu was bound to Gel through a bond involving the carboxyl and amino groups. These results showed that Flu and Gel were bound *via* electrostatic interactions, as we expected, because Flu is negatively charged at approximately neutral pH and that positively charged basic Gel was used in this study.

The present characterization of Flu release from atelocollagen/gelatin complexes showed that the sustained Flu release rate decreased with increasing the AC content. Concentration of Flu in this experiment is different for each complex. AC is soluble only in an acidic solution. On the other hand, Flu is precipitated in the acidic conditions. Therefore, it was impossible to add Flu in the dissolved state in AC solution. Maximum amount of Flu, which was capable to bind with each Gel solution, was mixed to Gel for preparation of F-AC. In our preliminary experiment, Flu was confirmed not to bind to AC. The complex prepared from Gel alone (F-AC0) was found to release 90% of Flu in 1 day. Previous reports have documented that the release of a physiologically active substance from a carrier occurs in two stages (*i.e.*, carrier diffusion and erosion), which can occur in steps or simultaneously^{33,34}. Similarly, it is likely that statin diffusion from F-AC0 and disintegration of the sponge itself concurrently occurred in our experiment, resulting in the rapid elevation of the release rate. In contrast, complexes containing a low proportion of AC (F-AC10 and 20) released $\geq 50\%$ Flu in 1 day with a subsequent gradual increase of the release rate. Complexes with high AC content (F-AC30 and 40) gradually released Flu over 7 days without rapid release in an early phase that was observed for other materials. Based on previous reports, these observations may be explained as follows. The initial release of a physiologically active substance caused by diffusion of a carrier, time required for transition to the decomposition-driven second stage of the release, and the release rate in that stage depend on the crosslink density of the carrier^{33,34}. Other researchers have shown that the strength of collagen can be enhanced by crosslinking³⁵, similar to that of gelatin. Therefore, it is likely that the release properties of Flu were prolonged with increasing the AC contents and the degree of crosslinking.

The present *in vivo* study showed that the sponge prepared of Gel alone (AC0) remained visible for 3 days at the implant site and disappeared 5 days after implantation. However, in animals that received AC40, AC remained visible for 5 days after

surgery and disappeared completely on day 7. These results suggest that, unlike the pure Gel sponge, the atelocollagen/gelatin complex was not absorbed *in vivo* in an early stage following the implantation and may support sustained drug release for an extended period. Controlled release of Flu *in vivo* is believed to take place appropriately after inflammatory phase. Further investigations are necessary to examine optimal concentration of Flu and AC/Gel ratio *in vivo*.

In summary, this study showed that atelocollagen/gelatin complex had porosity similar to conventional carriers and confirmed bonding between Flu and Gel. The Flu release test showed that the release rate decreased as the AC content increased. These results suggest that novel atelocollagen/gelatin complex may be clinically useful carriers to accomplish local sustained Flu release for acceleration of bone formation. Therefore, the present study showed that sustained Flu release can be controlled using an atelocollagen/gelatin complex, suggesting the utility of this composite material.

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