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

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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 been extensively studied for possible applications, such as accelerating healing at extraction sockets and implant sites2,3).

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
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 µM Flu was dissolved to 10 mL of the phosphoric acid (0.9% Gel solution with Flu) according to the early study (18). 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 4ºC. 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 µM, 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 −20ºC for 2 h. Frozen samples were placed in a freeze dryer (FREEZONE®2.5, LABCONCO, Kanzas city, MO, USA) and dried under vacuum at −50ºC for 24 h to prepare dry sponges. Finally, atelocollagen/gelatin complex were polymerized by cross-linking the sponge with heat treatment at 125ºC 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 (1,600–1,500cm⁻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 37ºC. After sampling 60 µL 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 0.9% AC solution (%) | B 0.9% Gel solution with Flu (%) |
|-------------------------|------------------------|---------------------------------|
| F-AC40                  | 40                     | 60                              |
| F-AC30                  | 30                     | 70                              |
| F-AC20                  | 20                     | 80                              |
| F-AC10                  | 10                     | 90                              |
| F-AC0                   | 0                      | 100                             |
| AC/Gel complex without Flu | A 0.9% AC solution (%) | C 0.9% Gel solution without Flu (%) |
|-------------------------|------------------------|---------------------------------|
| AC40                    | 40                     | 60                              |
| AC30                    | 30                     | 70                              |
| AC20                    | 20                     | 80                              |
| AC10                    | 10                     | 90                              |
| AC0                     | 0                      | 100                             |
of eluate collected in each section/absorbance of initial F-AC solution of each complex)×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±1°C with 60±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 µL/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% 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 µm-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 (Axiohot 2, Carl Zeiss, Oberkochen, Germany).

Statistical analysis
All data are expressed as the mean±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 µm.

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 1,600–1,500 cm⁻¹ range, in which marked peak changes are observed. The peak at 1,587 cm⁻¹, a carboxyl group-
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
Fig. 5  Histological analysis of absorption of complexes of AC0 (a–c) and those of AC40 (d–f) in femoral at day 3 (a, d), 5 (b, e), and 7 (c, f).
In animals that received AC0, Gel sponge residues were observed on postoperative day 3 (a). No sponge structure was visible on postoperative day 5 or 7 (b, c). In animals that received AC40, residual AC fibers intensively stained with hematoxylin were observed on postoperative day 3 (d). The residual fibers were yet visible on postoperative day 5, and they were completely absorbed on day 7 (e, f). Scale bars: 200 µm.

DISCUSSION
In the present study, we prepared and characterized atelocollagen/gelatin complexes as carriers for controlled, sustained statin release.

In our earlier studies, we used a Gel sponge carrying Flu via electrostatic interactions\(^{18-20}\). The dissolution rate of this Gel sponge is controllable for up to 3 days by thermal crosslinking\(^{20}\). However, no conventional thermal crosslinking methods were effective in further prolonging the dissolution time of the Gel sponge. AC used in this study, from which antigenic telopeptides have been removed by proteolytic treatment, is highly biocompatible and has been widely used in research and clinical settings\(^{22}\). Although collagen and gelatin are identical to each other in primary structure (amino acid composition), they differ in secondary structure, and collagen, which forms gelatin on partial hydrolysis, has lower susceptibility to proteases than gelatin\(^{21}\) because of its three-dimensional structure. Therefore, atelocollagen was adopted as a biodegradable carrier with a reduced rate of dissolution, even without undergoing harsh crosslinking treatment, which is required for gelatin use. However, the fibrillar protein collagen has a hydrophobic character and was considered to be incompatible with the electrostatic interaction-based method used to attach Flu in our earlier study. Although other studies have used simvastatin-impregnated atelocollagen sponge (Teruplug\(^{9,14,23}\)), these reports do not describe statin binding by collagen or characterize the time course of statin release. In the present study, we accordingly prepared an atelocollagen/gelatin complex in which Gel was used as a carrier of Flu, as in our earlier studies, and AC was used to retard the degradation of the carrier.

When gelatin is used as a carrier, the solubility is controlled by the rate of crosslinking. Crosslinking is accomplished using chemicals such as formaldehyde\(^{24}\) and glutaraldehyde\(^{25}\) or physical methods such as UV irradiation\(^{26}\) and heating\(^{18-20}\). However, it has been reported that with chemical crosslinking, cytotoxicity of residual reactants from the chemical treatment causes issues\(^{24,25,27}\). Because nontoxic, thermally-crosslinked gelatin was successfully used in our earlier studies, we used thermal crosslinking in the present study.

SEM observations showed no marked structural differences among complexes prepared from F-AC0 (a conventional Gel sponge) and F-AC10, 20, 30, and 40, with a porous structure consisting of approximately 200 µm pores observed for all analyzed materials. The
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 Flu carboxyl group was absent and the peaks at 1,557 and 1,546 cm⁻¹ from the amino group of Gel 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 250% 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 crosslinking[35], 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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