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Effect of collagen fibril formation on bioresorbability of hydroxyapatite/collagen composites

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Abstract Porous hydroxyapatite/collagen (HAp/Col) composite is a promising biomaterial and a scaffold for bone tissue engineering. The effect of fibril formation of Col in the porous composite on bioresorbability and mechanical strength was investigated. The fibril formation, in mixing a self-organized HAp/Col nanocomposite and sodium phosphate buffer at a neutral condition, occurred during incubation at 37 °C, resulting in gelation of the mixture. The porous composites with and without the incubation were obtained by freeze-drying technique, in which macroscopic open pores were formed. The compressive strength of the porous composite with the incubation (34.1 ± 1.6 kPa) was significantly higher than that without the incubation (28.0 ± 3.3 kPa) due to the fibril formation of Col. The implantations of the porous composites treated with a dehydrothermal treatment in bone holes revealed that bioresorption was clearly depended on the fibril formation. The bioresorbability in vivo was almost matched to the in vitro test using enzymatic reaction of collagenase.

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Introduction

Bone tissue engineering has been recently focused in orthopaedic surgery to substitute traditional bone-defect treatments including autograft [1–3]. The scaffolds with three-dimensional (3-D) porous structure are critical for bone tissue engineering to promote cell and/or tissue ingrowth while maintaining the transport of oxygen and nutrition [3]. The scaffolds based on inorganic and organic substances such as calcium phosphates (hydroxyapatite (HAp), tricalcium phosphates and biphasic calcium phosphates), and synthetic and biological polymers (PLA, PLLA, PGA, collagen (Col) and chitosan etc.) have been investigated [4]. Bone is composed of HAp nanocrystals and Col with a higher ordered structure and the HAp/Col composites have, thus, been extensively studied as bone regeneration materials [5–11]. Kikuchi et al. have developed the self-organized HAp/Col nanocomposite with similar nanostructure to bone tissue [12]. They fabricated the porous HAp/Col composite by using gelation of the nanocomposite including added Col molecules as binder, subsequent freeze-drying and chemical crosslinking.

Bone regeneration materials require the control of bioresorbability depending on the implanting location and the size of bone defects, of which characteristic should be also important for the scaffolds. Furthermore, the materials in vivo are always exposed to the mechanical stress such as compression, tension and torsion; therefore, the materials should have a shape recover property with sufficient porosity.

The bioresorbability and mechanical property of Col-based biomaterials are controlled by crosslinking techniques; physical treatments such as ultraviolet (UV) irradiation and dehydrothermal treatment (DHT), and chemical treatments such as glutaraldehyde and water soluble carbodiimide [13]. Chemical treatments confer low bioresorbability and remarkably high mechanical strength, but may also result in potential cytotoxicity. The physical treatments have no potential cytotoxicity. The fibril formation of Col [14, 15] also affects the bioresorbability and improvements of the mechanical strength [16], which also influence the cell attachment, proliferation and migration properties.

The aim of the present study is to investigate the effect of fibril formation of Col in porous HAp/Col composites on the bioresorbability and mechanical strength. The mechanical property of the porous composites with and without the fibril formation was evaluated using compression tests. The bioresorbability of the porous composites were analyzed by in vitro and in vivo tests: the weight loss after soaking in the enzyme solution and implant into the bone holes of rat.

Materials and methods

Synthesis of HAp/Col nanocomposites

The HAp/Col nanocomposite was synthesized according to our previous method [17]. About 400 mM of $Ca(OH)_2$ suspension and 120 mM of H_3PO_4 including Col molecules (type I atelocollagen from porcine dermis; Nitta Gelatin, Japan) were simultaneously added to distilled water at 40 °C. The concentration and amount of the starting substances: Col, phosphoric acid, and $Ca(OH)_2$ were selected to obtain a HAp/Col weight ratio of 80/20. The pH was controlled to be between 8 and 9; at this pH, the formation of Col fibrils and HAp nucleation occurred in a nano-region. The nanocomposite suspension obtained was washed with distilled water and freeze dried.

The HAp/Col weight ratio in the freeze-dried nanocomposite was quantified using thermogravimetry and differential thermal analysis (TG-DTA Thermo Plus TG 8120; Rigaku, Japan) at temperatures ranging from room temperature to 1200 °C in static air at the heating rate of 20 °C/min.

Gel strength of HAp/Col composite mixture

The freeze-dried HAp/Col nanocomposite was kneaded in water or 100-mM sodium phosphate buffer (pH 6.8) at a concentration of 1.25 g/10.0 mL [18]. The mixtures were pored into a 24-well biological dish to be the height of 10 mm, and incubated at 37 °C. The mechanical strength (mean ± SD; $n = 2$) was measured by a texture analyzer (TA-XT2i; Stable Micro Systems, U.K.) without removal of the gel from the biological dish. The cylindrical probe (Φ5.0 mm) was inserted into the gel at the cross-head speed of 0.1 mm/s (strain rate of 1.0%/s) to a breaking point, at which the stress was adopted as the gel strength.

Preparation of porous HAp/Col composite

The HAp/Col mixture described above was put into a polystyrene container (25 × 25 × 14 mm) and subjected to two treatments as follows:

- With incubation: set in an incubator at 37 °C for 24 h and subsequently in a freezer at –20 °C.
- Without incubation: set in a freezer at –20 °C.

The completely frozen mixtures were freeze dried. The porous HAp/Col composites obtained were dehydrothermally treated at 140 °C for 12 h in vacuo to introduce cross-linkages among the Col molecules. In this study, the porous composites prepared with and without incubation were abbreviated to be HAp/Col*i* and HAp/Col*wi*, respectively. Their porosities were estimated from the weight and volume of the cylindrical specimens (Φ8 × 8 mm) ($n = 6$), where the specific densities of HAp and Col were assumed to be 3.16 and 1.00 g/cm^3.

Mechanical property

Mechanical property of the porous HAp/Col composites were evaluated by the compression tests using the texture analyzer according to our previous method [18]. The porous HAp/Col composites were cut into a cylindrical shape (Φ8 × 8 mm), soaked in PBS for 24 h, and then subjected to the compression tests. The Student's *t*-test was used to access the statistical significance of the results ($p < 0.01$).

SEM observation

The morphology of Col in the HAp/Col mixture incubated at 37 °C for 24 h was observed by SEM (JEOL-5600LV; JEOL, Japan). The mixture was fixed with glutaraldehyde and, then demineralized completely in 200-mM EDTA/20-mM Tris-HCl buffer (pH 7.5). The Col residue was dehydrated by ethanol and subsequent t-butanol, and dried in vacuo at 0 °C where the frozen t-butanol sublimates. The SEM observation was operated at the accelerating voltage of 10 kV. The cross-sectional morphology of the porous HAp/Col composites was also observed.

In vitro bioresorbability

The blocks of the porous composites with 52.8 ± 7.3 mg (mean ± SD) in weight were immersed in 5 mL of PBS containing 500 units of bacterial collagenase (*Clostridium*

histolyticum), and incubated at 37 °C for certain periods. The value of collagenase concentration was employed to complete the tests within 24 h to eliminate the effect of bacterial proliferation on the bioresorbability. The external solution including degraded scaffolds was removed using a pipette. The remained blocks were frozen, freeze dried, and then weighed ($n = 3$ for 2, 4, and 8 h, and $n = 6$ for 12 and 18 h). The weight in certain periods was normalized as the remaining weight (%) against the initial weight. The Student's *t*-test was used to access the statistical significance of the results ($p < 0.01$).

In vivo bioresorbability

A bone hole (Φ 3 × 3 mm) was made in the femur of male Wistar rats (age, 10 weeks; weight, 280–320 g) according to a previous report [19]. HAp/Col*wi* and HAp/Col*i* (Φ 3 × 3 mm) were inserted into the bone holes ($n = 3$). For weeks after the implantation, the implants were cut out from the implanted site. The tissues were fixed with 4% of paraformaldehyde, decalcified in 20% EDTA, dehydrated through graded alcohol series, cleared in xylene, and embedded in paraffin wax. Serial sections of 5 μm thickness were cut and stained with Hematoxylin and Eosin (HE).

Results and discussion

The HAp/Col nanocomposites freeze-dried had the weight ratio in the range from 80.5/19.5 to 80.2/19.8 as a result of TG-DTA analysis. In order to prepare the porous composites from the nanocomposites by freeze-dry technique, it requires homogeneous mixture of the nanocomposite prior to the freezing process. The promotion of Col aggregation in the nanocomposites makes it difficult to prepare sponge-like porous composites, due to the lack of homogenous Col dispersion in the composites. Thus, we synthesized the nanocomposite under the condition of remarkable inhibition of Col aggregation at the high ionic concentration [20]. The synthesis under this condition provided the self-organized HAp/Col nanocomposites with smaller size compared to those prepared at the lower ionic concentrations. Figure 1 shows the compressive strength of the HAp/Col mixture with phosphate buffer at each incubation time. The compressive strength gradually increased with the incubation time. After incubating the mixture at 24 h, the gel strength showed almost plateau, of which tendency was almost similar to the curves of pure Col fibril formation.

Figure 2a and d shows the SEM images of the demineralized HAp/Col nanocomposites mixtures. The mixtures prepared from water (data not shown) and from sodium phosphate buffer without the incubation (Fig. 2a) had no fibril formation. On the other hand, the demineralized

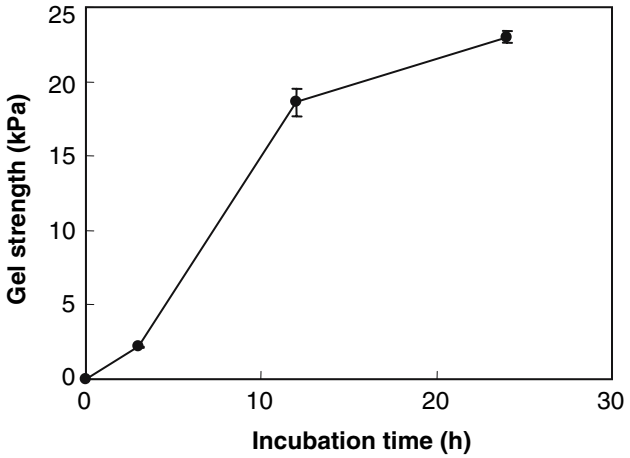

Fig. 1 Gel strength of HAp/Col nanocomposite mixture with sodium phosphate buffer against incubation time at 37 °C

nanocomposite/buffer mixture after the incubation had a dense network of nano-fibers, in Fig. 2d, similar to the nanostructure of Col fibrils reconstituted in vitro [21]. The gelation of the nanocomposite/buffer mixture could be therefore attributed to the reconstitution of the Col in the self-organized HAp/Col nanocomposite, similar to the in vitro reconstitution of pure Col.

We successfully prepared a couple of porous HAp/Col composites: HAp/Col*i* and HAp/Col*wi*, stabilized by dehydrothermal treatments. Their respective porosity was determined to be 93.6 ± 0.1% and 93.8 ± 0.1% from the calculation of its weight and volume. Figure 2b and c show the cross-sectional SEM image of HAp/Col*wi*, in which the macroscopic pores with spherical shape were randomly distorted and the pore size was widely distributed. Although it was difficult to calculate the pore size distribution directly from the SEM images reported in Fig. 2, they should be enough for tissue engineering scaffolds in which the pore sizes in the range 200–300 μm are generally required [2]. The cross-sectional morphology of HAp/Col*i* (Fig. 2e and f) was almost similar; however the pore size distribution was slightly smaller.

The compressive strength of HAp/Col*i* (34.1 ± 1.6 kPa) was significantly higher than that of HAp/Col*wi* (28.0 ± 3.3 kPa). The slight different porosity and structure of both porous composite might partly contribute to the different strength; however, it appears that Col fibril formation plays a predominant role on the improvement of strength.

Figure 3 shows the weight loss of HAp/Col*i* and HAp/Col*wi* in the collagenase solution at 37 °C. The collagenase solution has been used to investigate in vitro bioresorbability of Col-based materials, because collagenase digestion is the initiation of the Col degradation in vivo. The weight loss of HAp/Col*i* was significantly smaller than that

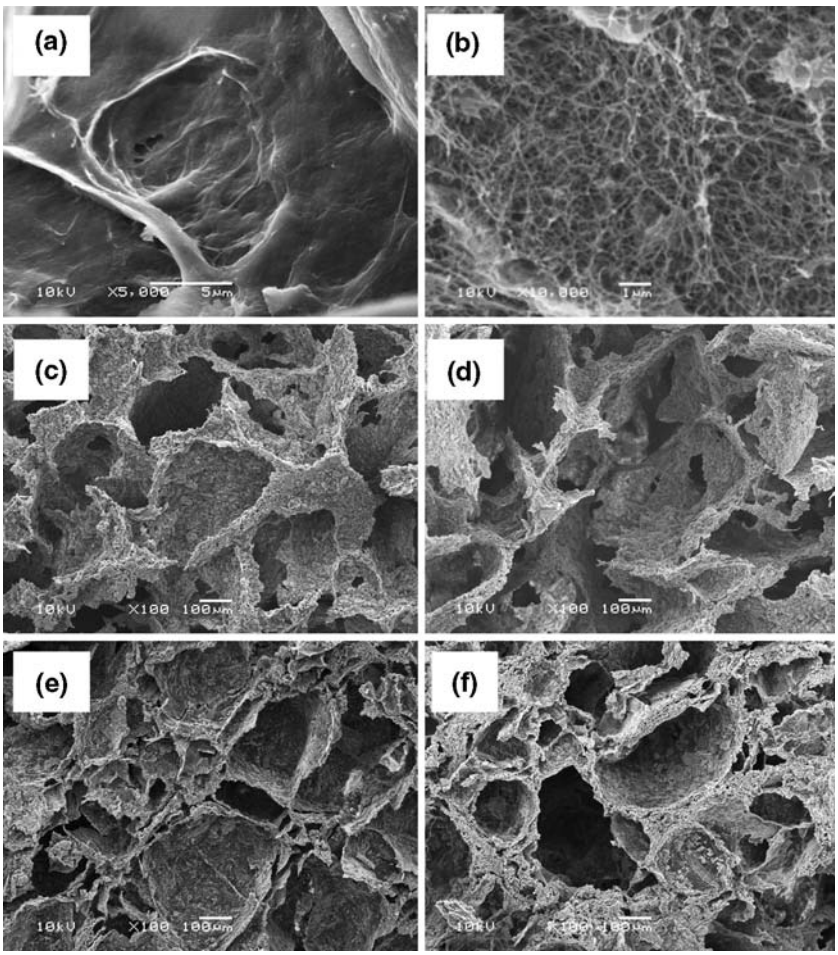

Fig. 2 SEM images of the demineralized mixtures of the HAp/Col nanocomposite and sodium phosphate buffer without incubation (**a**) and incubated at 37 °C for 24 h (**d**), and cross-sectional SEM images of the porous composites: HAp/Col*wi* (**b, c**) and HAp/Col*i* (**e, f**)

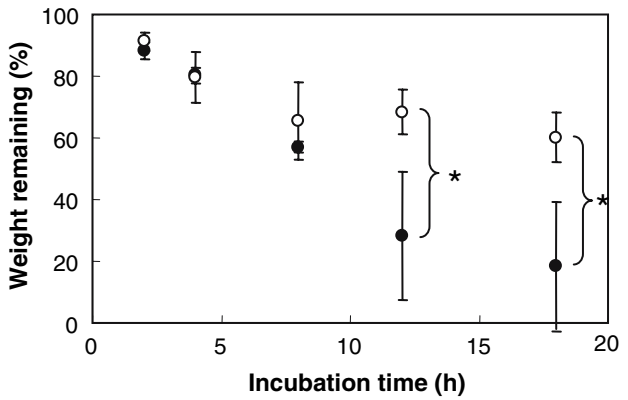

Fig. 3 Weight loss of HAp/Col composite after soaked in collagenase solution. Open circles, HAp/Col*i*; Closed circles, HAp/Col*wi*. Asterisks indicate the statistical significance between the two examinations ($p < 0.01$)

of HAp/Col*wi* after 6 h-incubation, suggesting Col fibril formation suppressed its bioresorbability.

Figure 4 shows the optical microscope images of the histological sections for the HAp/Col composites into the bone holes of rats at 4 weeks. In the implanted site, most of the HAp/Col*wi* implant was resorbed and the new bone formation clearly occurred (Fig. 4a). The pore structure in the implant was partially remained and filled with bone marrow. On the other hand, the bioresorption rate of the HAp/Col*i* implant was much lower than that of HAp/Col*wi* (Fig. 4b). Bone formation was observed only around the pores. It is known that the biological stability (i.e., resistance against enzymatic degradation) of Col based biomaterials can be significantly improved by the fibril formation of Col molecules [16]. The Col fibril formation therefore played an important role for the suppression of the bioresorption of the HAp/Col composite. These results indicate that the in vivo bioresorbability apparently supports the in vitro results for HAp/Col composites.

Conclusion

We successfully prepared the porous HAp/Col composites with or without Col nanofibrils by mixing the self-organized HAp/Col nanocomposite and sodium phosphate buffer at

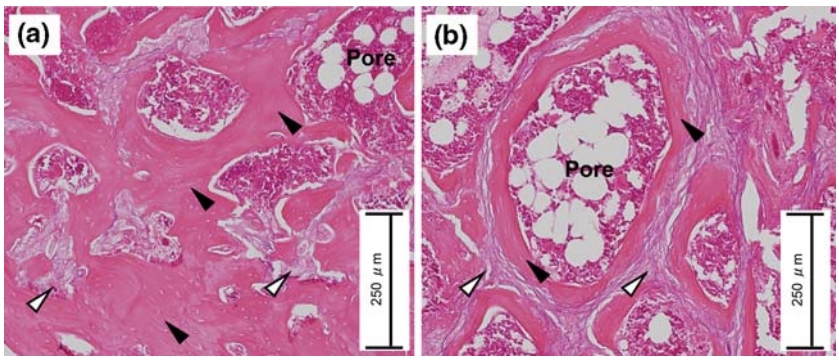

Fig. 4 Optical microscope images of the histological sections for the HAp/Col composites scaffolds implanted into the bone holes of rats at 4 weeks. (a), HAp/Col*wi*; (b), HAp/Col*i*. Open arrows indicate the implants remained. Closed arrows indicate the newly formed bone. The pore structure filled with bone marrow was clearly observed in the implanted site of HAp/Col*i*; on the other hand, those of the HAp/Col*wi* implant were only partially remained

neutral pH and subsequent freeze drying. Nano-fibril formation of Col occurred in the HAp/Col nanocomposite/buffer mixture by incubation at 37 °C. The in vivo and in vitro bioresorbability of the porous composites clearly indicated that Col fibril formation improved their mechanical property and reduced bioresorbability.

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