インジェクタブル自己硬化型水酸アパタイト／コラーゲン骨ペーストの作製

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博士学位請求論文

Fabrication of injectable and self-setting hydroxyapatite/collagen bone paste
(インジェクタブル自己硬化型水酸アパタイト/コラーゲン骨ペーストの作製)

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Chapter 1 Introduction

1. 1. Human bone

Our bone has two roles; one is a structural function that supports our body from load and protects important organs, a brain, heart and lungs, against impact. Another is a biochemical function to maintain calcium homeostasis. To realize these two roles, bone has unique chemical composition and nanostructure. The mass composition of our bones is 60 to 70 % of inorganic substance, non-stoichiometric carbonate-containing hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HAp) of 20 to 40 nm in length, 20 to 25 mass% of organic substances, mainly type I collagen of approximately 300 nm in length, and approximately 10% of water; *i.e.*, simplified description of bone is a nanocomposite of HAp nanocrystals and collagen molecules [1-3], with a primary aligned nanostructure of *c*-axes of HAp nanocrystals and elongation direction of collagen fibers [4]. Secondary structure of bone is a bundle formed by the five nanocomposite fibers. Tertiary structure is found in cortical bone, as an osteon, an annual-ring like structure. Each ring is nanocomposite membrane composed of aligned bundles and direction of bundles is different from the next rings [5]. Bone is constantly metabolized by the bone remodeling process that old and/or cracked parts of bones are resorbed by osteoclasts, bone resorbing cells, followed by formation of new bone by osteoblasts, bone forming cells. A bone resorption or bone formation is also play a important role on the calcium homeostasis.

1. 2. Artificial bone void filler

The bone remodeling process allows bone to self-repair small defects due to a disease or injury with appropriate treatments. However, defects exceeding the critical size of self-repair induce invasion of the scar tissue before bone formation and do not regenerate completely. Therefore, grafting of bone void fillers, to prevent invasion of scar tissue and to aid in regeneration of bones, is generally applied to repair large bone defects. Autologous bone grafting using the patient's own ilium and fibula is a gold standard due to its high effectiveness, because it transplants not only bone matrix but also cells, cytokines and other effective substances to support bone regeneration. Further, it does not show rejection and is eventually incorporated into bone remodeling process to substitute with new bone. Even it seems to have no bad points, it still has

several problems, *i.e*., the need for secondary surgery in healthy parts to collect bones, restriction on the amount, size and shape of the collected bone, and side effects, mainly pain at the harvesting site after the surgery. Therefore, artificial bone void fillers have been being studied and applied to support autologous bone transplantation.

Conventionally, sintered HAp ceramics [6, 7] and bioactive glasses [8] are widely used because of their high osteoconductivity. However, even HAp nanocrystals in bone are resorbed rapidly by osteoclasts, absorption/resorption of these materials are very slow, and they remain for life-long of patients. Consequently, the remaining materials become a latent risk as a cause of secondary bone fracture in the implantation site, due to the difference in mechanical properties with bone. Therefore, the artificial bone void fillers using bioabsorbable materials to be replaced with living bone have been developed to the latent risk. One of the representatives of bioresorbable materials is β-tricalcium phosphate (β-$Ca_3(PO_4)_2$; β-TCP), which has approximately 2 times higher solubility than HAp for body fluids; however, the biodegradation mechanism of β-TCP is still unclear. [9] Moreover, a clinical study reported that no osteoclastic resorption of a large quantity of β-TCP was observed 72 weeks after grafting [10], even though porous β-TCP ceramics are used worldwide as bioactive and biodegradable bone void fillers [11].

1. 3. Biomimetic hydroxyapatite and collagen composite

To have biological reactions closer to autologous bone, researches on composite of HAp and collagen have been conducted to develop artificial bones, which have bone similar nanostructure and chemical composition.

In 2003, Olszta *et al*. synthesized bone-like apatite/collagen composite using a technique called polymer-induced liquid-precursor (PILP) method [12, 13]. In brief, $CaCl_2$ solution and K_2HPO_4 solution are mixed at slight supersaturation concentration of HAp, and a commercially available collagen sponge is immersed in the solution to fill the gaps of the collagen fibers with the calcium phosphate solution by capillary action and polyaspartic acid as a PILP. Finally, bone-like structures are reproduced by crystallization of the calcium phosphate. Apatite/collagen composite by this method has excellent bone mimicry [14]; however, it require long-time to form the composite and difficult to scale-up for commercial use.

In 2010, Nassif *et al*. reported synthesis of bone-like aligned composite of HAp

and collagen by raising pH of acidic mixture solution of calcium, phosphate and collagen with ammonia gas. Raised pH of the solution induces collagen fibrillogenesis with a simultaneous induction of HAp nanocrystals nucleation in the collagen matrix [15]. On the other hand, concentrations of phosphate and calcium salt cannot be increased, since the solubility of the calcium phosphate salt is generally low in the acidic solution in which collagen molecules are stable, and control of pH of whole reaction vessel homogeneously in large reaction vessel is quite difficult, thus, the method could be difficult to apply in large-scale synthesis for practical use. In addition, complete removal of ammonia should needs large amount of cold water. These reasons may restrict commercial use at this time.

In 2001, prior to above mentioned apatite/collagen composites, Kikuchi *et al*. synthesized a hydroxyapatite/collagen bone-like nanocomposite (HAp/Col) by a simultaneous titration method *via* self-organization between HAp nanocrystals and collagen molecules [16]. The simultaneous titration method is a simple method as written, that starting solutions, $Ca(OH)_2$ aqueous suspension and collagen-added phosphoric acid solution, are simultaneously titrated to a reaction vessel with a maintenance of the reaction temperature at the body temperature, *i.e.* approximately 37 ºC, and controlling pH at 9 in the vessel by addition of the starting solutions through tube pumps with an on/off control. The HAp/Col synthesized by these conditions have the target mass ratio of collagen/(HAp and collagen) from the ratio of starting materials, and its compact shows the highest three-point bending strength among various conditions. The temperature is generally considered as good for collagen fibrillogenesis, and the pH is for HAp, basic calcium phosphate, stable formation as well as a point of zero charge of collagen. Accordingly, collagen fiber formation and HAp nucleation on carboxyl groups on the collagen fibers are simultaneously occurred. Directional relation between the HAp nanocrystals and carboxyl group on the collagen fibers is restricted by their chemical bonding, *i.e.*, *c*-axis of HAp is perpendicular to COO^- face of carboxyl group. Carboxyl groups on the collagen molecule can rotate to any direction but when two or more carboxyl groups bonded to surface calciu3m of one HAp nanocrystal, *c*-axis of the HAp nanocrystal and long axis of collagen molecule become almost parallel. In addition to that, HAp works as adhesive for collagen molecules, because ion concentration of the reaction solution is too low to occur collagen fibrillogenesis [17]. From another point of view, the carboxyl group in collagen induces nucleation and

precipitation of HAp, even though the optimal ion concentration for HAp/Col self-organization is lower supersaturated environment to the homogeneous HAp nucleation. In other words, HAp and collagen in the HAp/Col complement each other in self-organization.

The obtained HAp/Col has, therefore, a bone-like nanostructure and chemical composition and is resorbed by osteoclasts followed by new bone formation by osteoblasts, *i.e*. the HAp/Col is completely incorporated into the bone remodeling process. The simultaneous titration method has advantages in applications that no by-product nor possible remaining of toxic substances, such as ammonium in Nassif's report, are present in the product, synthetic equipment is simple, mass synthesis is easy, and the length of the composite fiber is controllable. The HAp/Col has attracted attention from the viewpoint of tissue regeneration and biomimesis, and its applied research has been conducted [18-20]. Among them, the porous body of the HAp/Col is commercially available as Refit® in Japan. In human clinical trials, the Refit® showed high bone regeneration ability in comparison to commercially available β-TCP porous body, Osferion® [21]; therefore, the HAp/Col is a superior bioresorbable material in terms of a rapid substitution with bone.

1. 4. Injectable bone paste

Bone void fillers have variety of shapes to correspond to variety of cases, *i.e*, dense body, porous body, granule and paste. Dense bodies are applied to comparatively load-bearing site and porous bodies are applied to introduce cells and tissues in its body. Granules are filled in various void shapes easily. Paste type fillers are most interesting materials and reduce invasion of surgical operation site as well as completely fit to bone defect. In other words, the paste is an important form enabling effective treatment that cannot be implemented with other material form. Clinically available bone pastes in present are fabricated by mixing of dicalcium phosphate [$CaHPO_4$] and tetracalcium phosphate [$Ca_4(PO_4)_2O$: TeCP] (Ceratouch®), [22] or α-tricalcium phosphate [α-$Ca_3(PO_4)_2$: α-TCP] and TeCP (Biopex®). The main reactions in each curing are respectively shown in Formula 1-1 and 1-2.

$$2Ca_4(PO_4)_2O + 2CaHPO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 \quad (1\text{-}1)$$

$$2\ Ca_3(PO_4)_2 + Ca_4(PO_4)_2O + H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 \quad (1\text{-}2)$$

These pastes utilize a hydration hardening as a hardening reaction, and the main component of the final products is HAp, which has risks mentioned in Section 1-2. Thus, a bioresorbable bone paste has been strongly desired by surgeons. Konishi *et al*. proposed chelate-setting hardening system for calcium phosphate powder using inositol hexaphosphate as a chelating agent and fabricated bioresorbable α-TCP and β-TCP self-setting cements as well as HAp cements. The α-TCP and β-TCP cements set remaining a TCP phase; therefore, these α-TCP and β-TCP cements were partly resorbed during 4 weeks observation and showed new bone formation in their bioresorbed areas [23, 24]. On the other hand, although the inositol hexaphosphate is used as an oral supplement, it has not yet been tested for any physiological reactions, including toxicity as a substance directly applied in internal tissues; therefore, it needs many biological tests to verify its use as a cement setting agent.

Utilization of suitable hardening agents and the HAp/Col as raw material of paste is considered to be one effective solution to respond to the surgeons' demand. Although the HAp/Col is reported as a dense body, a porous body, a granule, and a sheet, a practical self-setting injectable HAp/Col paste has not been reported at present. A HAp/Col paste cannot apply self-setting mechanism of the hydration reaction used in conventional bone cement; therefore, hardening agent is required for preparing it. For example, a method of hardening (gelling) HAp/Col fibers by gelling the added collagen has already been reported. However, this gelling method takes several hours at 37 °C to gelation of collagen, *i.e.* it does not solidify for several hours after injection into the body. Moreover, in order to fabricate the injectable HAp/Col by this method, cross-linking agent besides HAp/Col and collagen solution are required, and it has little strength after gelling; therefore, this approach is considered to be poor in practical use. As another approaches, sodium alginate (Na-Alg) which reacts with calcium ion or acid to gel and (3-glycidoxypropyl)trimethoxysilane (GPTMS) which is one type of silane coupling agents will be considered for hardening agent to prepare a HAp/Col paste.

1. 5. Overview of thesis

Aiming at fabrication of a paste type bone filling using HAp/Col, preparation of paste raw materials, condition of the hardening agents and mixing ratio of HAp/Col

and hardening agent were examined, and then the properties of the prepared HAp/Col pastes were investigated. In chapter 2, the optimum mixing conditions of HAp/Col paste using Na-Alg were determined. Furthermore, the influences of additives of organic acid or Ca compound were investigated. In chapter 3, in order to obtain anti-washout property for the HAp/Col–Na-Alg paste, the influence of Ca compound addition was examined in further detail. In chapter 4, preparation of paste using GPTMS was performed and its physical properties were investigated. In chapter 5, a biological investigation of HAp/Col-GPTMS paste was conducted. Finally, conclusion of this thesis was described in chapter 6.

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Chapter 2 Preparation of injectable hydroxyapatite/collagen paste using sodium alginate and influence of additives

Excellent biological functions of the HAp/Col derive from its nanostructure and chemical composition; thus, conventional hardening systems of HAp bone pastes, based upon *in situ* formation of HAp, are not suit for the HAp/Col because its nanostructure cannot be formed under the hardening systems. Consequently, gradual crosslink of collagen in the HAp/Col with a biocompatible crosslinker and/or fix the HAp/Col powder in a biocompatible and biodegradable hydrogel are possible candidates for preparation of the HAp/Col paste. Collagen is one of the best hydrogel in biological properties but its handling and long gelation time are big demerits for clinical applications. Gelatin, a denatured product of collagen, also has high biocompatibility and gelation abilities; however, gelatin gel cannot be used because it changed into sol at body temperature and temperature control of gelatin sol not to denature collagen molecules in the HAp/Col is also difficult at bed side.

Contrarily, sodium alginate (Na-Alg) has rapid gelation in appropriate calcium salt solution and used for scaffold of regenerative medicine in many researches [1-4]. The Na-Alg is an acidic polysaccharide contained in marine algae such as kelp, has biocompatibility and biodegradability An alginic acid has a linear structure and is composed of randomly aligned mannuronic acid (M) and guluronic acid (G) monomers. The blocks where G are arranged consecutively form a chelate so called “egg-box structure” by capturing multivalent cations including Ca ions to induce gelation of Alg [5]. This gelation improved anti-decay property of apatite cement in the blood [6] and would give the same effect on the HAp/Col paste. In addition, the Na-Alg has another gelation property via precipitation of alginic acid gel by extrusion of Na^+ in an acidic environment. Further, Alg gel has a biodegradability by exchange of multivalent ions in gel and Na^+ ions in living tissue.

In this chapter, the optimal conditions of preparing HAp/Col–Na-Alg paste was investigated for injection, and the HAp/Col–Na-Alg paste of optimal conditions was added several kinds of Ca-salt or organic acid as hardening (gelling) accelerator for improving pastes' anti-washout property. The HAp/Col powder of 100-212 μm was prepared from the HAp and collagen mass ratio of 80:20 that was fabricated by the simultaneous titration method, and the HAp/Col paste was then prepared by mixing of

the HAp/Col powder and the Na-Alg aqueous solution at several powder/liquid ratios. The paste optimal conditions for injection were determined by results of viscosity test, hardening behavior test and compressive strength test; however, the HAp/Col–Na-Alg paste with optimal conditions immersed in phosphate buffered saline was decayed within few hours. Therefore, Ca compound or organic acid was supplemented to the paste of optimal conditions in order to improve the paste anti-washout property, and properties of the additive supplemented pastes were investigated.

2.1. Materials and methods

2.1.1. Materials

The HAp/Col with the HAp and collagen mass ratio of 80:20 was prepared by the simultaneous titration method [7, 8]. The brief preparation procedure for 10 g of the HAp/Col was that 100 cm^3 of 400 mM $Ca(OH)_2$ (prepared from alkaline analysis grade $CaCO_3$ (Wako Pure Chemicals Inc., Japan) suspension and 200 cm^3 of 120 mM orthophosphoric acid (Reagent grade, Wako Chemicals Inc., Japan) solution with 2.0 g of type-I porcine dermal collagen (Biomaterial Grade, Nitta Gelatin Inc., Osaka, Japan) solution were simultaneously titrated via tube pumps to the reaction vessel, in which 100 cm^3 of pure water was previously added, with maintaining of temperature at 37 °C and pH at 9. The obtained HAp/Col was analyzed by the powder X-ray diffractometry (XRD, Rigaku, RINT-Ultima III) and thermogravimetry-differential thermal analysis (TG-DTA, Rigaku, Thermo Plus, Japan). The obtained HAp/Col was compacted to a disk of 32 mm in diameter and 2 mm in height using a specially designed mold for squeezing water by uniaxial pressing at 20 MPa, and freeze-dried. The HAp/Col disk was then crushed into 100-212 μm in size, and collagen molecules in the HAp/Col powder were dehydrothermal-crosslinked at 140 ºC for 12 h under vacuum. Primary hardening reaction was expected to be gelation of Alg with Ca^{2+} ions released by partial dissolution of HAp nanocrystals in the HAp/Col. However, Sotome *et al.* reported that the HAp/Col adsorbed Ca^{2+} and Mg^{2+} ions in the cell culture medium [9]. It means that competitive reactions, chelating of Ca^{2+} ions by Alg and adsorption of Ca^{2+} ions on to HAp/Col, would occur during paste hardening and may decrease the hardening. To minimize the influence of Ca^{2+} ions adsorption by the HAp/Col powder, Ca-adsorbed HAp/Col (Ca-HAp/Col) powder, the HAp/Col powder with saturated adsorption of Ca^{2+}

ions on it, was prepared by stirring the HAp/Col powder in 20 mM $CaCl_2$ solution for 3 days, followed by filtering, freeze-drying and re-sieving to collect powders 100-212 μm in size. The HAp/Col and Ca-HAp/Col powders were observed with a scanning electron microscope (SEM, JSM5600LV, JEOL, Japan). Sodium alginates used in the experiment were 80-120 (L-Na-Alg), 300-400 (M-Na-Alg), 500-600 cP (H-Na-Alg) in viscosity at 10 g/dm^3 (Wako Pure Chemicals Inc.) The pastes were prepared by mixing of the Na-Alg solution with HAp/Col or Ca-HAp/Col powder under conditions summarized in Table 2-1. To confirm if the amount of Na-Alg was enough, the prepared paste was directly injected into 100 mmol/dm^3 $CaCl_2$ (Wako Pure Chemicals Inc., Japan) aqueous solution, this amount is usually enough to form gel by injecting Na-Alg solution, using syringe (Thermo, 2 mm in caliber). The noodle-like paste was harvested from solution after one minute soaking and observed their conditions by naked eye and fingers.

Gelation of Alg by egg-box formation via Ca^{2+} would be an essential factor to control setting time and anti-decay property of the paste. Release amounts of Ca^{2+} can be controlled by an organic acid to dissolve HAp in the HAp/Col or by Ca compounds with optimal solubility. Therefore, possible candidates for additive would be an organic acid. An organic acid additive was chosen from citric acid (tricarboxylic compound), succinic acid, malic acid (dicarboxylic compounds with different carbon chain length), lactic acid and glycolic acid (monocarboxylic compounds with different carbon chain length.) Amounts of organic acid were 0.1, 1 and 10 mass% of HAp/Col according to the preliminary experiment to determine acceptable amount of lactic acid to maintain injectability of Na-Alg solution. A Ca compound additive was chosen from $Ca(CO)_3$, calcium citrate (Ca-Cit), $Ca(OH)_2$ and $CaSO_4 \cdot 0.5H_2O$ (ordered in small to large solubility.) Amounts of Ca compound were 0.5x, 1.0x and 2.0x of equivalent reaction amount (1.67 ± 0.07 mmol per 1 g of Na-Alg) of Ca ion to Na-Alg preliminary measured. These chemicals for the additive were purchased from Wako Pure Chemicals Inc. The additive except for lactic acid, provided as liquid, was added in a powder form.

The Ca-HAp/Col paste, which prepared at the Ca-HAp/Col to the L-Na-Alg mass ratio of 90:10 and the Ca-HAp/Col powder to the Na-Alg solution mass ratio of 0.60, was used to investigate influences of an additive. One additive chosen from Ca compounds was added to the Ca-HAp/Col paste while mixing.

The Ca-HAp/Col paste, which prepared at the Ca-HAp/Col to the L-Na-Alg mass ratio

Table 2-1. Mass concentration of Na-Alg solution at each conditions each condition.

(HAp/Col)/Na-Alg weight ratio	P/L ratio					
	0.82	0.69	0.60	0.53	0.47	0.43
95/5	4.8	4.0	3.5	3.0	2.7	2.4
90/10	9.1	7.7	6.7	6.7	5.3	4.8
80/20	16.7	14.3	12.5	11.1	10.0	9.1

of 90:10 and the Ca-HAp/Col powder to the Na-Alg solution mass ratio of 0.60, was used to investigate influences of an additive. One additive chosen from Ca compounds or organic acids was added to the Ca-HAp/Col paste while mixing.

2.1.2. Viscosity and hardening behavior tests

Viscosity of the prepared paste was measured according to Ishikawa et al. [10]. Briefly, 0.1 cm^3 of the paste was mixed for 3 min and a 2 kg glass plate was placed on the paste in 10 min after the start of mixing. The spread area was then measured at 10 min after placing the glass. According to the preliminary test, a hardening behavior test described in the Japanese industrial standard JIS T 6602 for dental zinc phosphate cement could not be applied for the HAp/ Col and Ca-HAp/Col pastes because of softness of the pastes. Thus, a hardening behavior of the paste was investigated by timedependent viscosity test. The 0.3 cm^3 samples, to distinguish a change of value, were packed in silicone tube mold, and were incubated for 1, 2, 3 and 4 days in an incubator maintained its temperature at 37 °C and relative humidity of 100%. The tested pastes were prepared under conditions summarized in Table 2-2. In addition, the influence of additive on initial hardening behavior was measured using Ca-HAp/Col paste with 2.0 x Ca- Cit and that with 10 mass% succinic acid to Ca-HAp/Col powder at 1, 6 and 24 h after incubation by the viscosity test.

2.1.3. Compressive strength test

The samples were prepared according to Table 2-2. The paste was packed in a silicon tube mold (5 mm inside diameter, 6mm in height), placed in an incubator for 1, 2, 3 and 4 days. The compressive strength of the paste was then measured with a texture analyzer (TA-XT2i, Stable Micro Systems Inc.) with plunger of 10 mm diameter and head speed of 1mm/min.

2.1.4. Decay property test

Decay property for the paste was measured by the procedure in Japanese industrial standard JIS T 0330-4 Bioceramics-Part4: Characterization of calcium phosphate paste. In detail, after 3 min mixing of raw materials, the mixed paste was packed in the syringe of 4.8 mm in inner diameter and 16.5 mm in height. Within 5 min after the start of mixing, the paste was squeezed on wire net with wire diameter of

Table 2-2. Powder/liquid ratios for hardening behavior and compressivestrength testseach conditions.

Molecular weight of Na-Alg	Mass ratio of HAp/Col to Na-Alg
	90:10
Low	0.60*
Middle	0.53
High	0.53

* for hardening behavior

0.5 mm and aperture of 2.0 mm, and was soaked into 50 cm^3 of 37 °C phosphate buffered saline (PBS). The paste in PBS was then statically placed at 37 °C for 72 h in an incubator. Decay rate was calculated from the final weight of paste left on the net, and decaying time for the paste that completely decayed within 72 h was also measured.

2.2. Results

As shown in Fig. 2-1, no significant differences of particle morphology between HAp/Col and Ca-HAp/Col powders were observed with SEM images as well as specific surface areas of them, 55.1 ± 3.7 $m^2 \cdot g^{-1}$ for the HAp/Col and 58.2 ± 3.8 $m^2 \cdot g^{-1}$ for the Ca-HAp/Col powders. The paste poured into $CaCl_2$ aqueous solution hardened immediately as shown in Fig. 2-2(a), and that collected from the solution had enough strength to handle in surgical and cell culture operation with viscoelasticity as shown in Fig. 2-2(b).

Figure 2-3 shows results of viscosity test for various conditions. Viscosity increased (spread area decreased) with increasing in P/L ratio and/or increasing in viscosity of Na-Alg used.

Figures 2-4 and 2-5 shows the results of viscosity test for the paste prepared with an organic acid and a Ca compound, respectively. Effect of additive on viscosity depended on its amount. At the mass ratio of HAp/Col or Ca-HAp/Col to Na-Alg 95: 5, the prepared paste was very fragile at 1 day after incubation due to insufficiency of Na-Alg mount to fix the paste.

The time-department changes in viscosity of the HAp/Col and Ca-HAp/Col pastes at Na-Alg ratio of 10 mass% are shown in Fig. 2-6. Viscosities of both pastes increased with time until day 2; however, that of the HAp/Col paste reached plateau at day 3 even that of the Ca- HAp/Col paste was continuously increasing at least till day 4. Figure 2-7 shows the hardening behavior as a function of time for the paste prepared with 10 mass% succinic acid or 2x Ca-Cit.

Figure 2-8 shows the results of compressive strength test. Compressive strengths of all pastes increased with time with similar behavior except for the Ca-HAp/Col paste prepared with L-Na-Alg. The Ca-HAp/Col paste prepared with L-Na-Alg only showed drastic increase of the compressive strength at 3 days after incubation than others.

On the decay property test, all pastes without the additive decayed completely

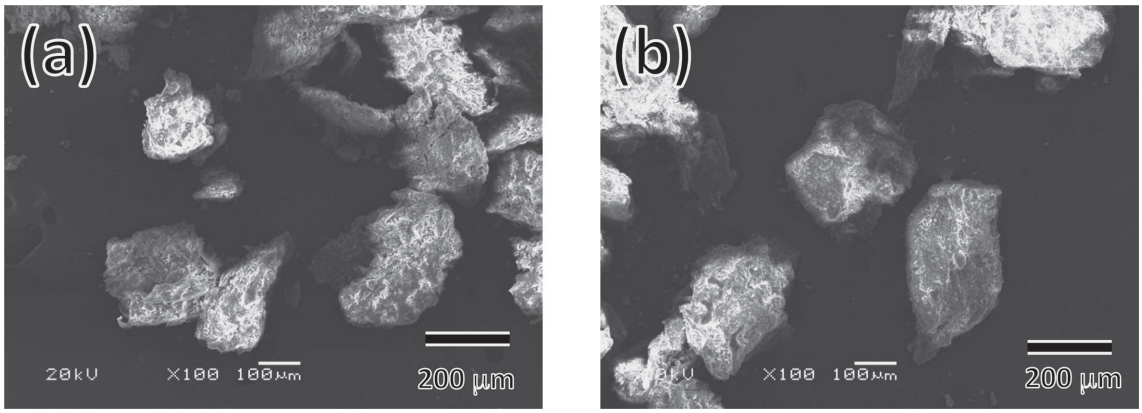

Fig. 2-1 SEM image of (a) HAp/Col and (b) Ca-HAp/Col.

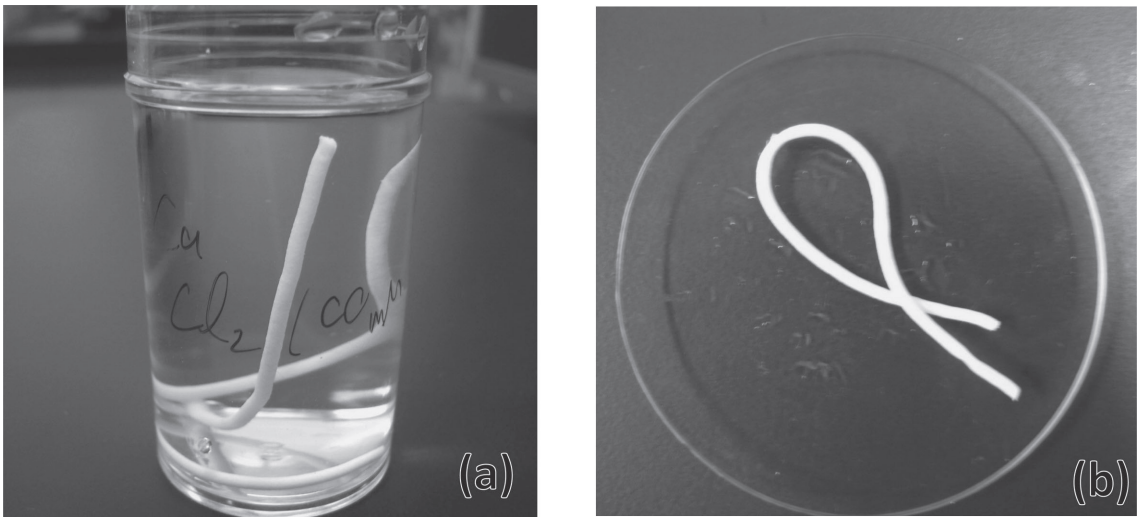

Fig. 2-2 (a) Ca-HAp/Col paste just after injected into 100mM $CaCl_2$ solution. (b) Ca-HAp/Col paste after immersed 100mM $CaCl_2$ solution for 1 min.

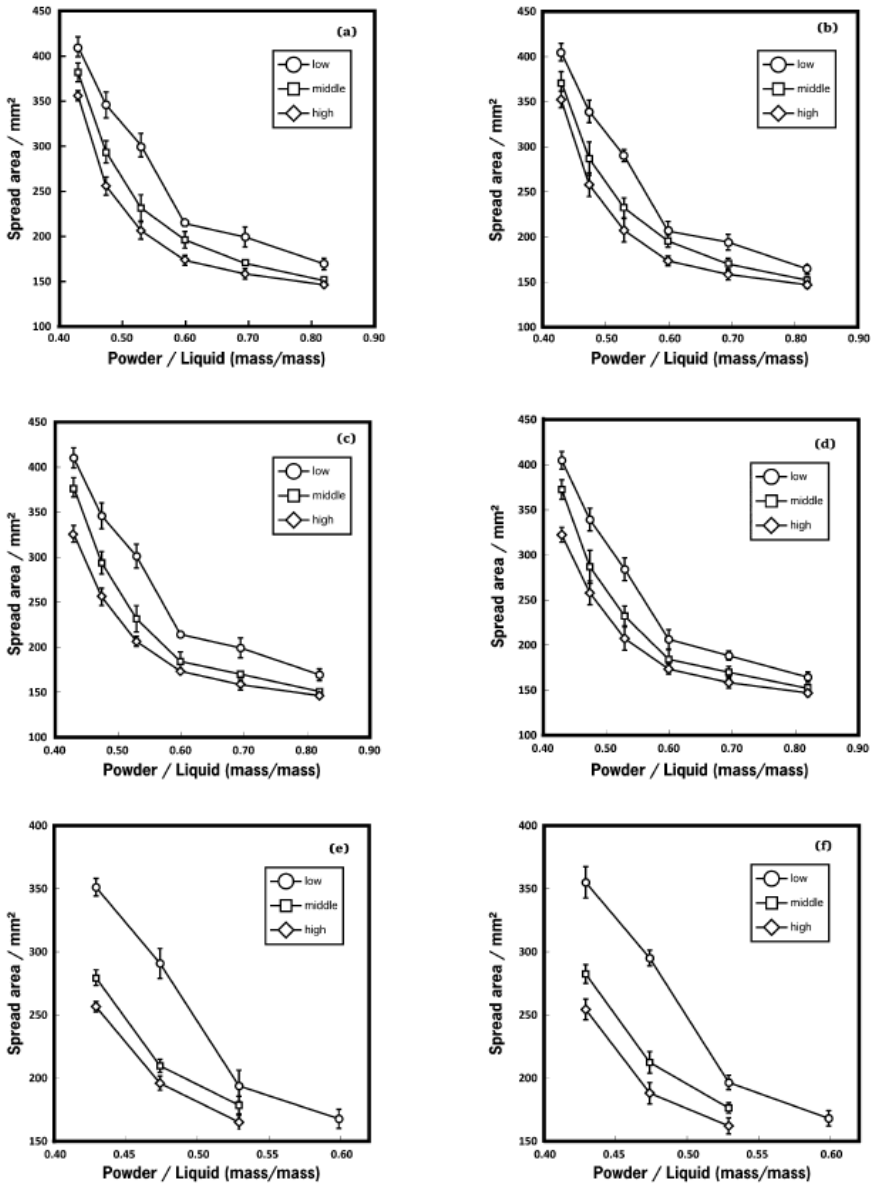

Fig. 2-3 Spread area, as an index of consistency, of the Hap/Col and Ca-Hap/Col paste prepared by under various conditions. Mass ratio of Hap/Col or Ca-Hap/Col to Na-Alg was fixed at 95/5, (a) Hap/Col and (b) Ca-Hap/Col paste. Mass ratio of Hap/Col or Ca-Hap/Col to Na-Alg was fixed at 90/10, (c) Hap/Col and (d) Ca-Hap/Col paste. Mass ratio of Hap/Col or Ca-Hap/Col to Na-Alg was fixed at 80/20, (e) Hap/Col and (f) Ca-Hap/Col paste.

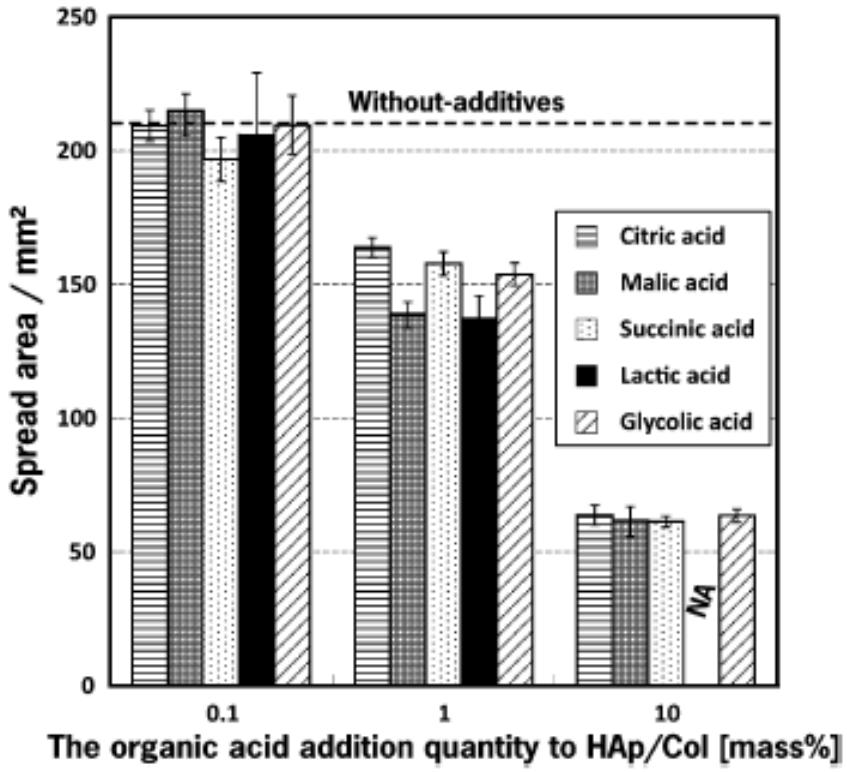

Fig. 2-4. Spread area of organic acid added Ca-HAp/Col paste.

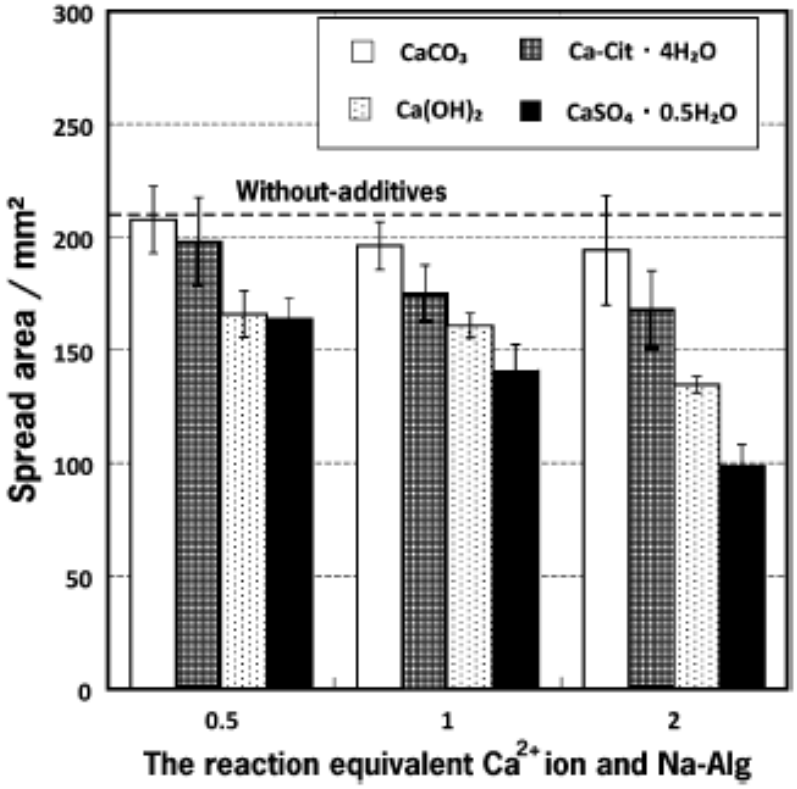

Fig. 2-5. Spread area of calcium compound added Ca-HAp/Col paste.

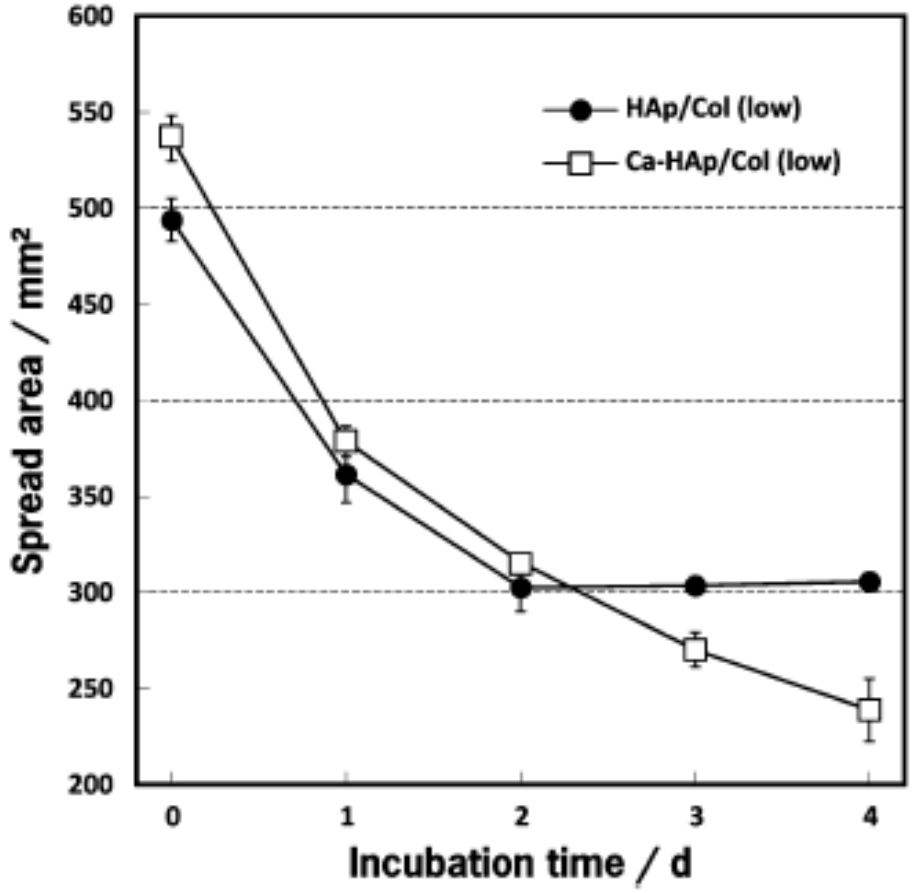

Fig. 2-6. Spread area of the HAp/col and Ca-HAp/Col pastes prepared at optimum P/L ratio as a function of incubation time. Mass ratio of HAp/Col or Ca-HAp/Col to Na-Alg was set at 90/10.

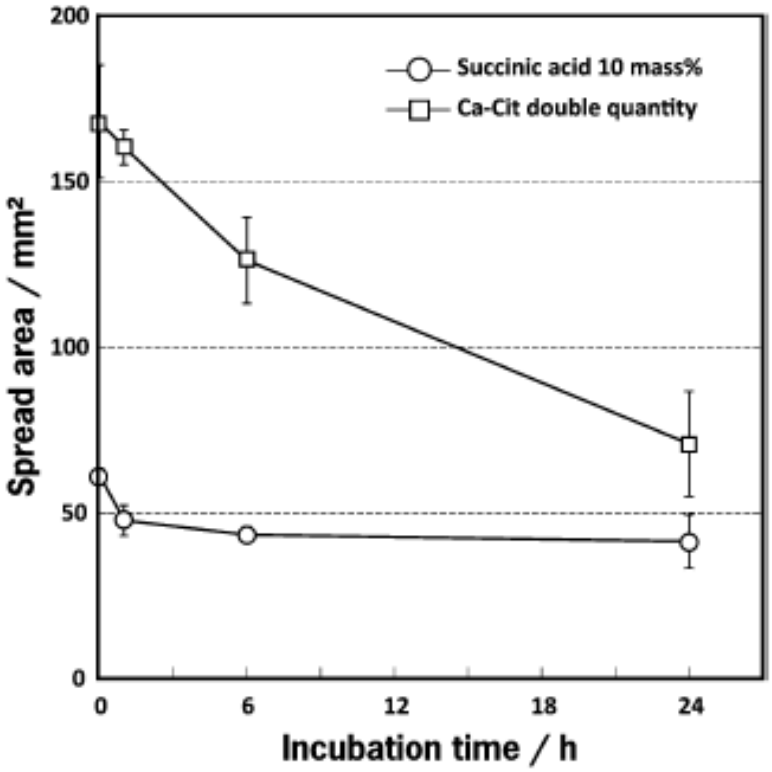

Fig. 2-7. Hardening behavior of additive-added paste.

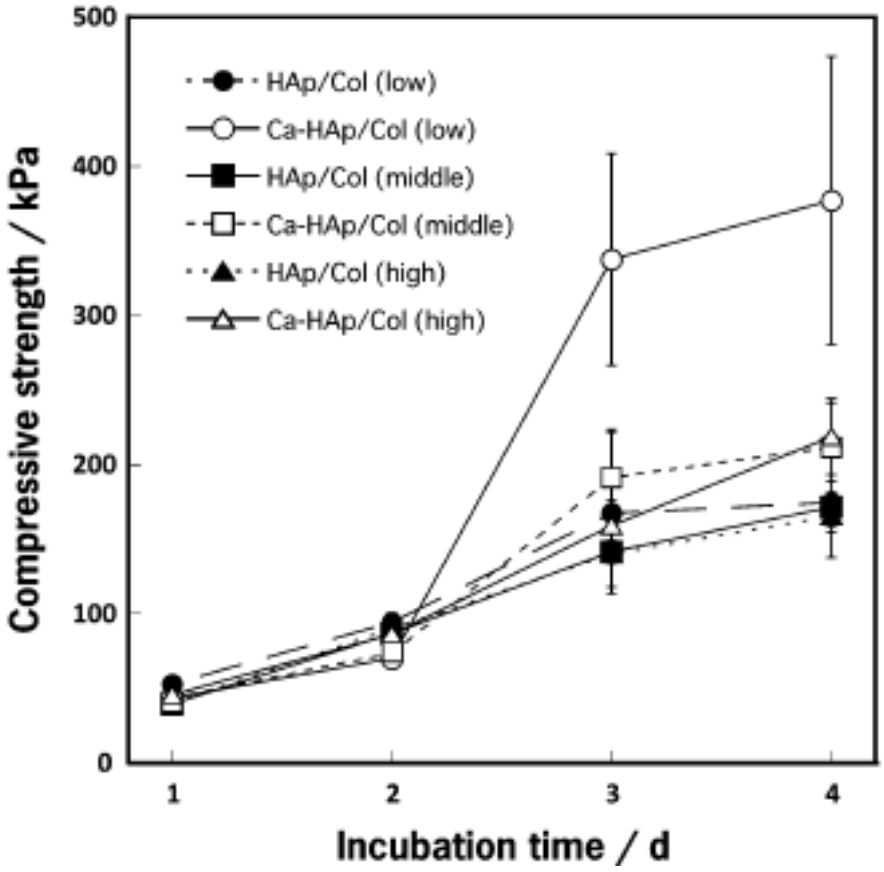

Fig. 2-8. Changes in compressive strength of the HAp/Col and Ca-HAp/Col pastes prepared at optimum P/L ratio as a function of incubation time. Mass ratio of HAp/Col or Ca-HAp/Col to Na-Alg was set at 90/10.

in the D-MEM in less than 2 h, and all pastes with the additive decayed within 24-48 h. Figure 2-9 shows decay time of the paste with Ca compound as the additive. Even viscosity of the paste prepared with $CaSO_4 \cdot 0.5H_2O$ or $Ca(OH)_2$ increased greater than that with other Ca compounds, no significant differences in decay time were observed between the paste with and without additives. Except for them, decay time showed the same trend to the viscosity change as well as Ca compound solubility.

2.3. Discussion

From the SEM observation and specific surface area measurement, treatment of Ca^{2+} ion on the HAp/Col did not affect apparent conditions of HAp/Col powder. Since the paste directly poured into $CaCl_2$ aqueous solution hardened immediately, 10 mass% of Alg in the paste was enough to cross-link via Ca^{2+} ions. In addition, this property is expected to apply the paste to rapid prototyping system for preparing fine controlled tissue engineering scaffolds. The most suitable P/L ratio of each pastes are determined as Table 2-3, the conditions that spread area revealed 200 mm^2, because the paste had good unity and had sufficient but not superabundant water content to allow injection.

The paste with at the mass ratio of 5 % of Na-Alg was very fragile at 1 day after incubation due to insufficiency of Na-Alg amount to fix the paste. Other Na-Alg ratio seemed to be good; however, to maximize the HAp/Col properties, we concluded that 10 mass% of Na-Alg would be much better than 20 mass% of Na-Alg. Although viscosity of the Ca-HAp/Col was continuously increasing at least till day 4, the HAp/Col paste reached plateau at day 3. These differences could be caused by difference in adsorption behavior of Alg on the HAp/Col and Ca-HAp/Col. Amounts of adsorption site, Ca, on the Ca-HAp/Col particles could be greater than those of HAp/Col, because of Ca^{2+} ion adsorption treatment. Initial stage of mixing, large amounts of Alg were adsorbed on the Ca-HAp/Col particles, subsequently amounts of free Alg for gelation decreased from liquid phase of the Ca-HAp/Col paste. In the meantime, surface of HAp nanocrystals in both paste started dissolution by chelating effect of Alg. Some of Alg molecules chelating Ca^{2+} ion(s) started to cross-link via egg-box structures. At this time, lower amounts of free Alg molecules in liquid phase of the Ca-HAp/Col paste allowed formation of smaller amounts of cross-links in comparison to the HAp/Col paste. As a result, viscosity of the Ca-HAp/Col paste was always smaller than that of the HAp/Col paste by day 2. Furthermore, Alg molecules

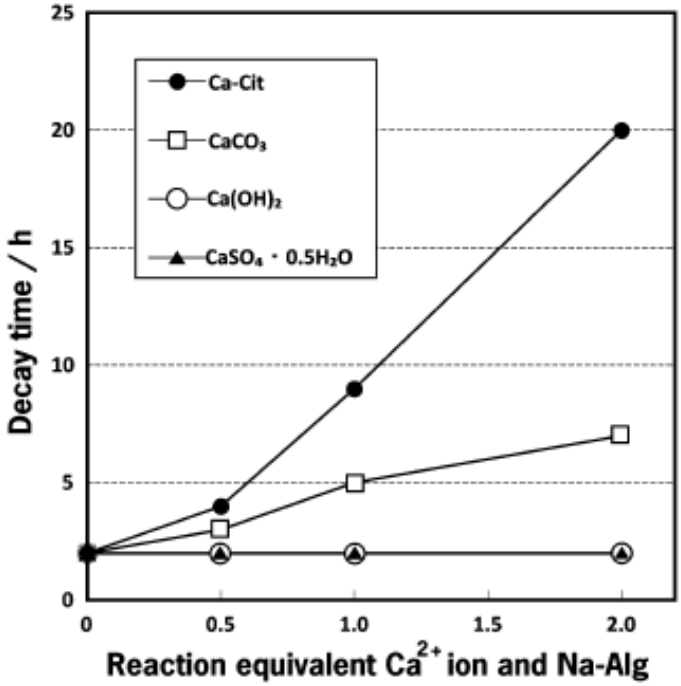

Fig. 2-9. Decay time of the Ca-HAp/Col pastes added calcium compound.

Table 2-3. Optimal P/L ratio for each condition.

Molecular weight of Na-Alg	Mass ratio of HAp/Col to Na-Alg		
	95:5	90:10	80:20
Low	0.69	0.60	0.53
Middle	0.60	0.53	0.47
High	0.60	0.53	0.47

easily cross-linked at neighborhood of the HAp/ Col particles due to existence of large amount of Alg molecules and could inhibit long range Alg gel network formation in the HAp/Col paste. Contrarily, gradual release of Alg molecules with Ca^{2+} ions from Ca-HAp/Col particle surfaces could form long-range network initially and form much harder gel in comparison to the HAp/Col paste with formation of stronger network at 3 or more days after mixing.

The Ca-HAp/Col paste prepared with L-Na-Alg only showed drastic increase of the compressive strength at 3 days after incubation. This phenomenon could be caused by differences in dispersion rates of Alg molecules in the pastes due to size of Alg molecules in combination with the usage of Ca-HAp/Col powder as described above.

For the organic acid added paste, number of carboxy group(s), size of molecules and/or chelate ability in organic acid had no influences on viscosity; thus, increase of paste viscosity could be only the influence of acid pH [5]. In the case of Ca compounds addition, the viscosity of the paste increased with increasing in both the amount and solubility of the Ca compounds. These results suggested that viscosity of the paste increased by the cross-link of Na-Alg in the paste by dissolved Ca^{2+} ions from the additive.

As a trend, viscosity of the pastes increased with time; however, the manner of hardening was very different between the pastes with succinic acid and Ca-Cit. With addition of succinic acid, the paste pH would decrease rapidly to introduce Alg gel formation by deposition of Alg from the Alg solution, then the paste viscosity decreased to approximately 60 mm^2 in spread are just after mixing and reached plateau, approximately 40 mm^2 in spread area in 6 h. Contrarily, viscosity of the paste prepared with Ca-Cit increased gently by slow release of Ca^{2+} ions from Ca-Cit; therefore, initial spread area was the same as that of the non-additive paste. Even though, the paste with Ca-Cit decreased its viscosity faster than that of the non-additive paste.

Fundamentally, Ca^{2+} release from the calcium compounds allowed to form Alg network faster than that released from HAp/Col; thus, viscosity of the paste increased with Ca^{2+} release rate, solubility. Contrarily, rapid Ca^{2+} release formed strong Alg gel surrounding of the Ca compound particles, and it inhibited dispersion of Ca^{2+} ions whole through the paste. Accordingly, Alg coagulates with Ca compound core formed in the paste to increase viscosity but to decrease unity of the paste, e.g., $CaSO_4 \cdot 0.5H_2O$

and $Ca(OH)_2$. In the present conditions, the paste composed of HAp/Col and Alg with non-decay property for an injectable artificial bone could not obtained; however, combination of organic acid and Ca compound would make the injectable artificial bone possible by control both initial viscosity to resist flow to accelerate decay and faster hardening via strong cross-link of Alg molecules via Ca^{2+} ions.

2.4. Summary

The optimal preparation conditions of the HAp/Col paste are that the P/L ratio is 0.6 with the 90:10 mass ratio of HAp/Col powder treated with $CaCl_2$ and low viscous Na-Alg. The prepared paste formed viscoelastic solid by direct injection into $CaCl_2$ aqueous solution. As an additive, organic acid increased paste viscosity very rapidly and Ca compound accelerate formation of egg-box cross-link in comparison to the non-additive paste. The pastes before hardened were decayed by soaking in D-MEM or PBS, some additive increased time to completely decay. In conclusion, the HAp/Col based paste prepared in this chapter would be applied in rapid prototyping with $CaCl_2$ aqueous solution soaking and could be a good candidate for injectable artificial bone with a property to completely incorporate into bone remodeling process after improvement of anti-decay property.

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Chapter 3 Influences of excess supplementation of calcium compounds on hydroxyapatite/collagen paste using sodium alginate

The chapter 2 describes preparation and characterization of injectable hydroxyapatite/collagen (HAp/Col) pastes utilizing sodium alginate (Na-Alg) as a lubricant and hardening agent, and the supplementation of small amount of any calcium compounds or organic acids did not improve the fast-setting and anti-washout properties sufficiently. In these calcium compound, insufficient but dose-dependent improvements were observed by supplementation of calcium compounds with a low solubility, *i.e.*, calcium carbonate and calcium citrate tetrahydrate (Ca-Cit). These results suggested that excess supplementation of these calcium compounds or combined supplementation of calcium compound and organic acid would be candidates of the HAp/Col paste which can be applied for practical use.

In this chapter, the influence of large amounts of supplementation of low soluble calcium compounds on the physical properties of the HAp/Col paste utilizing Na-Alg was investigated. Furthermore, the cytocompatibility of the HAp/Col pastes with large amount of Ca-Cit which obtained anti-washout was evaluated by a proliferation of human osteoblastic cell line, MG-63, cultured with the pastes.

3.1. Materials and methods

3.1.1. Materials

The powder phase of the HAp/Col pastes, HAp/Col powder, was prepared according to the previous chapter.

An aqueous solution of Na-Alg (80–120 cP in viscosity at 10 g/dm^3, Wako Pure Chemicals Inc., Japan, lot number DPN4907) at a concentration of 6.66 % in mass was used for the liquid phase of the HAp/Col. The concentration was determined from the optimum conditions from the previous report [1], i.e., the HAp/Col : Na-Alg mass ratio of 9:1 and powder/liquid (P/L) mass ratio of 1.00/1.67 (=0.60 mass/ mass). Calcium carbonate and calcium citrate tetrahydrate (Ca-Cit, $C_{12}H_{10}Ca_3O_{14}\cdot 4H_2O$; Reagent grade, Wako Pure Chemicals Inc., Japan) powder were used as supplements to provide Ca^{2+} ions to harden the paste. The amounts of Ca compound ranged from 0.5× to 20× the equivalent reaction amounts of the Ca^{2+} ion to Na-Alg (1.67 ± 0.07 mmol per 1 g of Na-Alg) preliminary determined by titration of a $CaCl_2$ solution into a Na-Alg

solution. The pastes were prepared by mixing a HAp/Col powder, one of the supplements, and Na-Alg aqueous solution under the conditions in Tables 3-1 and 3-2. Pastes supplemented with $CaCO_3$ and Ca-Cit were abbreviated as the $CaCO_3$-paste and the Ca-Cit-paste, respectively.

3.1.2. Viscosity test

The paste viscosity was measured according to 2.1.2. Ishikawa's paper [2]. As additional information, raw materials of the paste were mixed for 3 min, molded into a cylindrical shape with a 5.0 mm diameter and 5.1 mm high, which was approximately 0.1 cm^3 in volume, and placed on a glass plate. Its spread area was measured with the ImageJ (NIH, ver. 1.46, for mac) as the paste's viscosity using a digitally recorded image in the JPEG format with a digital still camera (μ-7040, Olympus Ltd, Tokyo, Japan) at 1920 × 2560 pixels.

3.1.3. Anti-washout property test

The washout property of the paste was measured in the same way as 2.1.4.

3.1.4. Cytocompatibility test

All the raw materials for the cytocompatibility test were sterilized with ethylene oxide gas. The HAp/Col pastes for the cytocompatibility test were chosen by the anti-washout property test followed by a preliminary soaking test in Dulbecco's modified Eagle's medium (Sigma-Aldrich, UK; D-MEM). They were molded into cylinders measuring 5 mm high and 7 mm diameter, and were aged to harden completely for 24 h in an incubator at 37 °C and 95 ± 5% RH. The cytocompatibility was tested with a human osteoblastic cell line, MG-63, using 6 cm^3 of the D-MEM supplemented with 10% fetal bovine serum (Cosmo Bio Co. Ltd., lot number 10D219) and 1% penicillin and streptomycin (Invitrogen-Gibco; Pen/Strep) as a culture medium. Twenty thousand cells were seeded in each well of a 6-well tissue culture polystyrene (TCPS) plate. 1 day after seeding, the culture medium was changed and a paste specimen was placed in each well. The medium was changed every 2 days, and the cell numbers were counted by on day 1, 3, and 7 after cell seeding with a hemocytometer. The used media were collected during the medium change to measure their Ca^{2+} and

Table 3-1 Mixing conditions of the HAp/Col paste with $CaCO_3$.

$CaCO_3$ X	0	0.5	1	2	5	10	20
HAp/Col / g	1	1	1	1	1	1	1
Na-Alg aq / g	1.67	1.67	1.67	1.67	1.67	1.67	1.67
$CaCO_3$ / g	0	0.009	0.019	0.037	0.093	0.185	0.371

Table 3-2 Mixing conditions of the HAp/Col paste with Ca-Cit.

Ca-Cit X	0	0.5	1	2	5	6	8	10	12	20
HAp/Col / g	1	1	1	1	1	1	1	1	1	1
Na-Alg aq / g	1.67	1.67	1.67	1.67	1.67	1.67	1.67	1.67	1.67	1.67
Ca-Cit / g	0	0.018	0.035	0.071	0.176	0.211	0.282	0.352	0.423	0.704

PO_4^{3-} ion concentrations with an inductively coupled plasma-atomic emission spectrometer (SPS7800, SII NanoTechnology, Japan; ICP-AES). The HAp/Col dense body, which was the same size as the pastes, was prepared by the same compacting method of the HAp/Col and die cutting using a punch followed by dehydrothermal cross-linking and soaking in the D-MEM for 5 days to adsorb Ca^{2+} and Mg^{2+} ions. A blank and the HAp/Col dense body were used as controls.

3.1.5. Statistical analysis

The results are shown as the mean ± standard deviation. Statistical analysis was performed using a one-way analysis of the variance with Tukey-HSD post hoc test. The statistical significance was set at $p < 0.05$.

3.2. Result

3.2.1. Viscosity test

Pastes prepared under all conditions were injected through a syringe with a 1.8 mm inner diameter. Their viscosities increased with the increase in the amounts of Ca compounds, but their increase behaviors were different, as shown in Fig. 3-1. The viscosities of the $CaCO_3$-pastes showed one-step and a slow increase with an increase in the $CaCO_3$ amounts and reached a plateau at 5× the amount. In contrast, the Ca-Cit-paste displayed a two-step increase in the viscosity. The first step, which occurred from 0× to 2×, rapidly increased compared to the $CaCO_3$-pastes but plateaued from 2× to 6× supplementations. The second step started from 6× with an increase rate similar to that of the $CaCO_3$-pastes, but slowed once the supplementation exceeded more than 10×.

3.2.2. Anti-washout property test

Improvements in the anti-washout property were found in both supplementations. However, critical differences were observed between the supplements. The 20× $CaCO_3$- paste decayed completely within 72 h and did not acquire a sufficient anti-washout property. In contrast, the Ca-Cit pastes had a drastic improvement in the anti-washout property, realizing a sufficient anti-washout property. Figure 3-2 shows the washout ratios of the Ca-Cit-pastes with an anti-washout property. Table 3-3 summarizes the pastes and pH of soaking PBS at the end of anti-washout test.

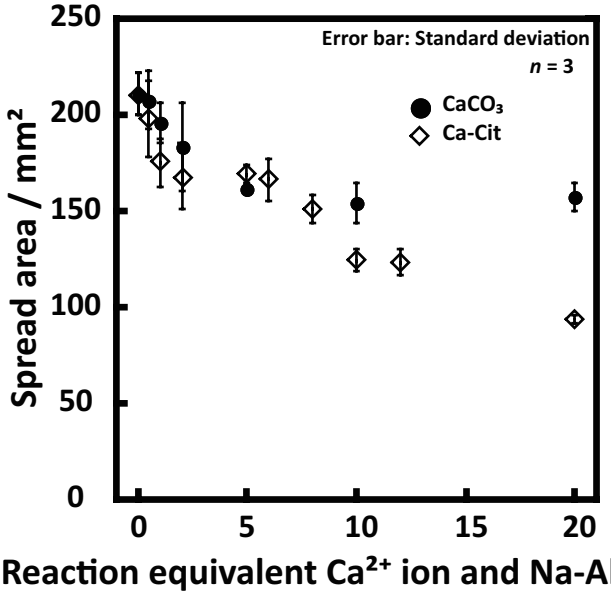


Fig. 3-1 Spread area of the HAp/Col paste with calcium compounds.

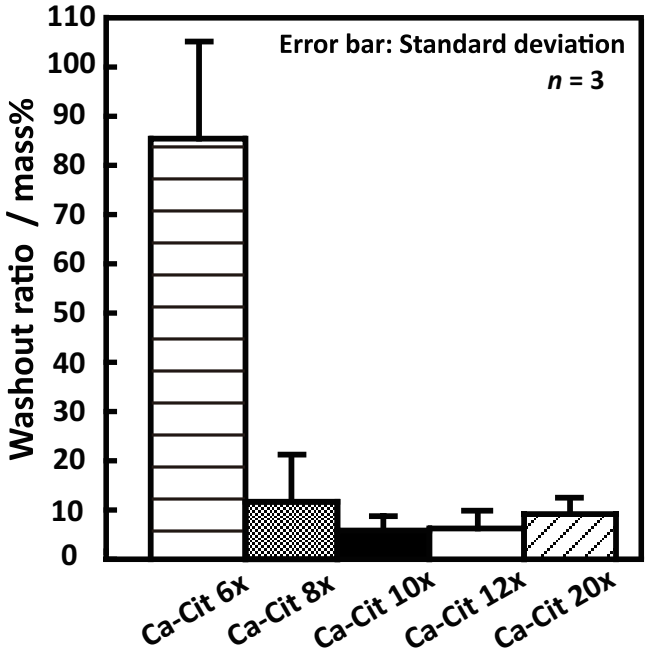
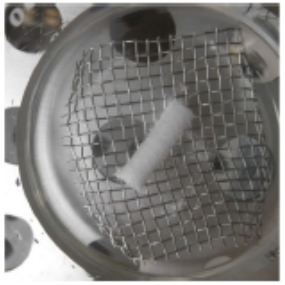


Fig. 3-2 Washout ratio of Ca-Cit added anti-washout HAp/Col paste.

Table 3-3 Final pH values of the soaking PBS and photos after the washout property test for the anti-washout pastes with Ca-Cit.

Ca-Cit ×	6	8	10	12	20
pH of PBS after test	6.70 ± 0.02	6.72 ± 0.13	6.72 ± 0.06	6.63 ± 0.05	6.64 ± 0.16
					

The 6× Ca-Cit-paste decayed largely, but the washout ratios of the 8× to 20× Ca-Cit-pastes were less than 10% in mass. These pastes maintained their original columnar shape during the test period. The 12× and 20× Ca-Cit-pastes removed from PBS after the anti-washout property test were covered with transparent gels as shown in Fig. 3-3a and b, respectively. This transparent gel was also observed, as shown in Fig. 3-3c, in the HAp/Col paste prepared with glycolic acid at 10% in mass after the anti-washout test performed in a previous report [1].

3.2.3. Cytocompatibility test

The anti-washout test indicated that the 8×, 10×, 12× and 20× Ca-Cit-pastes had potential as candidates for the cytocompatibility test. However, the 20× Ca-Cit-paste decayed in the preliminary D-MEM soaking test for 5 days. Thus, the 8×, 10× and 12× Ca-Cit-pastes were used for the cytocompatibility test. The cell proliferation curves are shown in Fig. 3-4. Significant differences in the cell number between each test group and the TCPS group were found in every measurement period. Significant differences were not found between each test group and the HAp/Col dense body group at the 7th day. Figures 3-5 and 3-6 plot the changes in the Ca^{2+} and PO_4^{3-} ion concentrations in the culture medium as a function of the culture period, respectively. At day 3, the culture media of the test group had a three times higher Ca^{2+} ion concentration than that of the TCPS control. In contrast, the culture media in which the HAp/Col dense body soaked showed a decrease in the Ca^{2+} ion concentration. The culture media of the test group and HAp/Col dense body group exhibited a lower PO_4^{3-} concentration compared to that of the TCPS. Additionally, the concentration of PO_4^{3-} ions continuously decreased with the culture period.

3.3. Discussion

The one-step and slow increase in the viscosities of the $CaCO_3$-pastes with an increase in the $CaCO_3$ amounts might be due to a restriction in the formation rate of the egg-box structure of alginate by the slow elution of Ca^{2+} ions from $CaCO_3$, which has a low solubility. In addition, the predicted weakly basic condition in the paste by dissolution of $CaCO_3$ did not affect the gelation of alginate. In contrast, the two-step increase in the viscosity of the Ca-Cit-paste could be caused by the following two types of alginate gelation system: the formation of the egg-box structure with a supply of Ca^{2+}

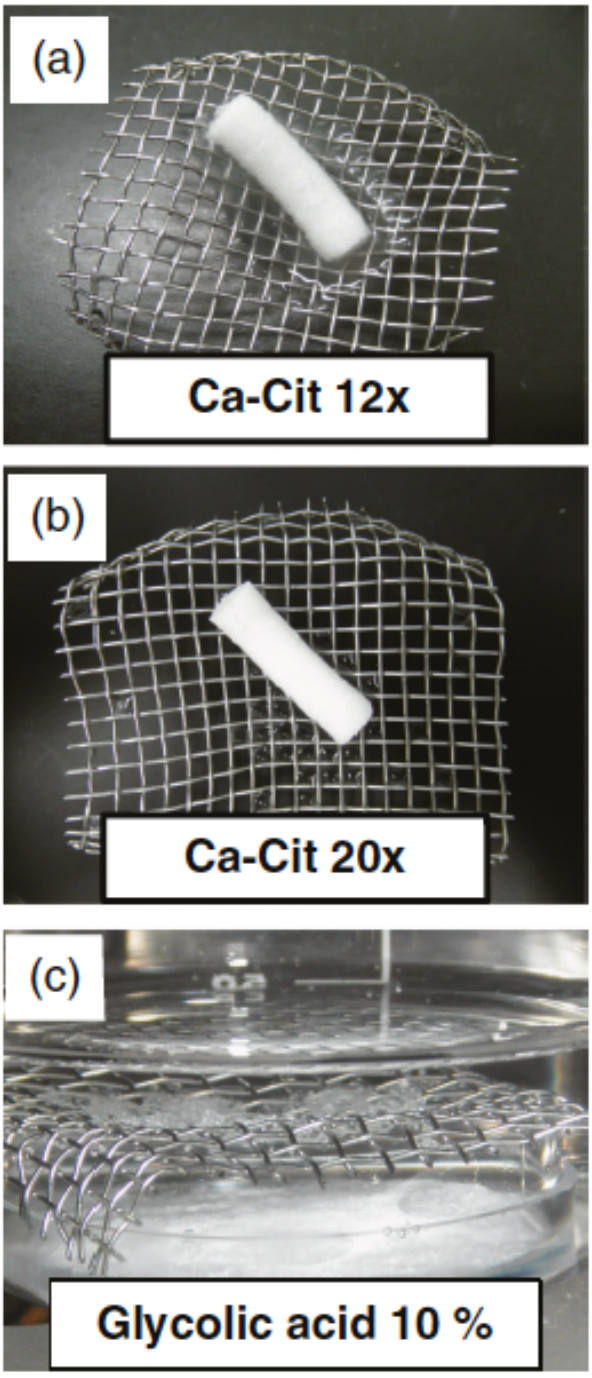


Fig. 3-3 Photos of the 12× and 20× Ca-Cit-pastes removed from PBS after the anti-washout property test (a, b). HAp/Col paste prepared with glycolic acid at 10% mass after the anti-washout test (c).

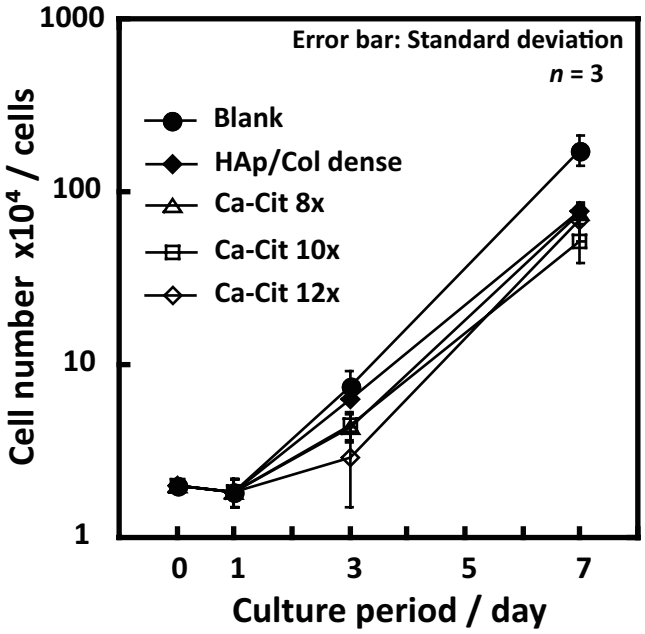


Fig. 3-4 Cell proliferation of MG-63 in the cytotoxicity test.

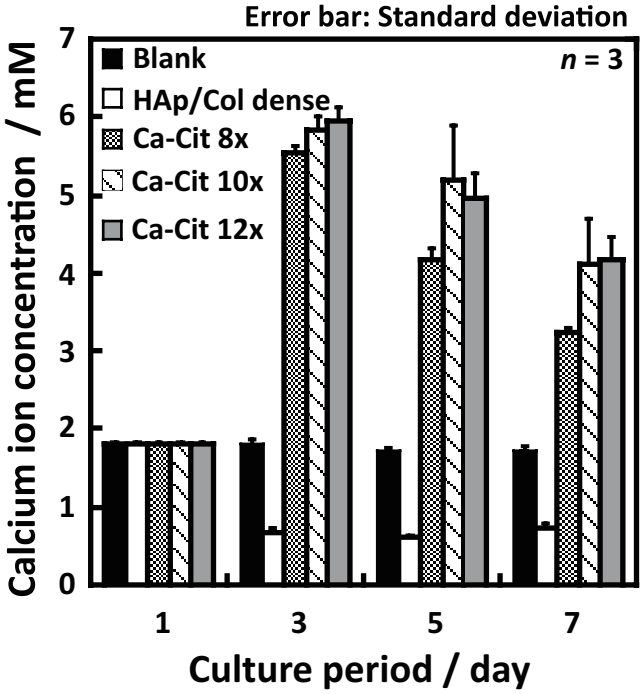


Fig. 3-5 Calcium ion concentration of the culture medium after the cytocompatibility test.

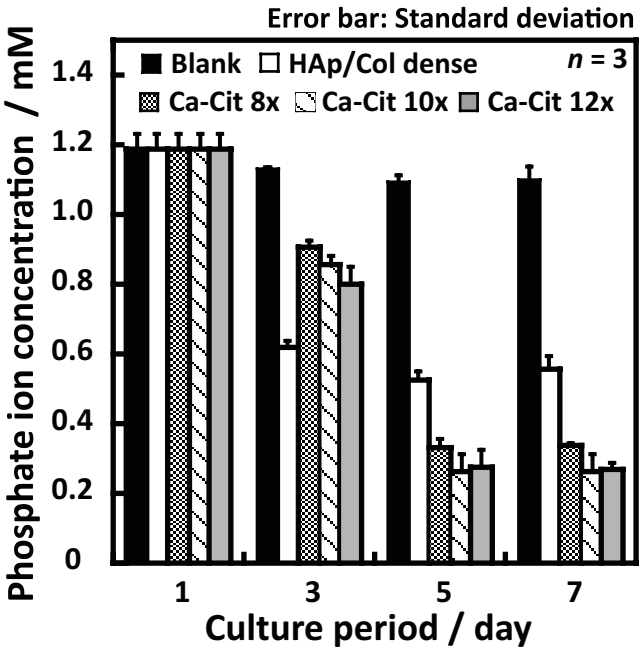


Fig. 3-6 Phosphate ion concentration of the culture medium after the cytocompatibility test

ions similar to the case of $CaCO_3$ supplementation or a change in the paste's pH to weakly acidic [3] due to dissolution of Ca-Cit.

The change in the pH in the paste was confirmed indirectly by the anti-washout test. The transparent gels observed in the anti-washout test of the paste prepared with organic acid were considered to be alginate gels formed by the acidic condition of the paste due to the presence of organic acids. Although transparent gels did not cover the 8× and 10× Ca-Cit-pastes in the observations by the naked-eye, an acidic pH of 6.7 of the PBS after the anti-washout test for these pastes might affect their biocompatibilities. Summarizing the anti-washout test results, supplementation of Ca compounds with a low solubility improved the paste anti-washout property by the gradual release of Ca^{2+} ions as assumed in the previous reports [1]. In addition, a complete anti-washout property was only observed in the 8× to 20× Ca-Cit-pastes, which showed alginate gelation by acidic pH. That is, the slow release of Ca^{2+} ion formed a weak but long-range gel. However, this weak gel easily decomposed by PBS soaking due to the exchange of Na^{+} in the PBS and the Ca^{2+} in the egg-box structure of alginate in the paste. On the other hand, alginate gel formed by an acidic environment might be stronger than the weak gel formed by small amounts of Ca^{2+} ions under the PBS condition, which coalesced the paste during the gradual egg-box structure formation by the large amounts of Ca^{2+} ion to reinforce the paste. For the reinforcement, the approximately ten times higher solubility of Ca-Cit than that of $CaCO_3$ could accelerate the reinforcement process.

The present Ca-Cit-pastes did not show significant differences in the cell number at the 7th day compared to the HAp/Col dense body group, indicating a good cytocompatibility. Additionally, the HAp/Col dense body showed a suitable biocompatibility in vivo. The culture media of the test groups had a Ca^{2+} ion concentration three times higher than that of the TCPS control at day 3 due to the gradual release of Ca^{2+} ions from Ca-Cit in the Ca-Cit pastes, but the ratio compared to the control decreased as the culture period increased. In contrast, the Ca^{2+} ion concentration of the culture media with the HAp/Col dense body decreased because Ca^{2+} ions in the media were adsorbed on the HAp/Col as reported by Sotome et al. [4]. The initial decrease in the PO_4^{3-} concentration was explained as an adsorption by the HAp/Col. The subsequent decrease was attributed to apatite formation on the surface of the HAp/Col or even the reaction that occurred partly in the initial stage. Changes in the

Ca^{2+} ions concentration in the medium affected the proliferation and differentiation of some cells [5], and a reduction in the PO_4^{3-} ions concentration inhibited proliferation for almost all cells [6]. Although both phenomena were observed in the test group in the present study, the inhibition of cell proliferation was very small and restricted. The cytocompatibility of the Ca-Cit pastes is as good as that of the pure HAp/Col, which is clinically used in Japan.

Furthermore, our colleagues reported that adding Ca-Cit and $CaCO_3$ in combination to the paste could adjusted the final solution pH of immersion liquid of washout property test to 7.4, which is the appropriate range of the cell culture medium, maintaining anti-washout property and cytocompatibility as well [7].

3.4. Summary

The pastes with excess supplementation of calcium compounds showed a gradual viscosity increase and injectability through a syringe with a 1.8 mm inner diameter to apply as injectable bone filler. The HAp/Col pastes showed a washout ratio at less than 10 % in mass by the addition of Ca-Cit at 8 or more times the equivalent reaction amount of Ca^{2+} ion to Na-Alg. All the anti-washout HAp/Col pastes showed a sufficient cytocompatibility; the MG-63 cells proliferated the same as the clinically available HAp/Col. Consequently, the HAp/Col injectable anti-washout pastes might be good candidates for bioresorbable bone filler pastes.

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Chapter 4 Preparation of anti-decay self-setting paste of hydroxyapatite/collagen utilizing (3-glycidoxypropyl)trimethoxysilane

In chapter 3, anti-decay property of the hydroxyapatite/collagen (HAp/Col) paste prepared with sodium alginate (Na-Alg) was sufficiently improved by adding calcium citrate (Ca-Cit). Meanwhile, Ca-Cit needs 30 mass% to the HAp/Col amount to acquire an anti-decay property, it may cause a decrement of HAp/Col advantages. Accordingly, preparation of HAp/Col paste with (3-glycidoxypropyl)trimethoxysilane (GPTMS), which is hardening agent substituting Na-Alg was researched. A GPTMS is one of the silane coupling agents which are commonly reported as functional materials to prepare biomaterials composed of inorganic or organic materials or their composites [1-3]. The silane coupling agents generally have the property of generating silanol groups by hydrolysis, and then they bond covalently to inorganic substances, and moreover, they form siloxane networks by their self-condensation [4]. In this study, GPTMS had been chosen because epoxy groups in GPTMS bond to amino groups in collagen [5–8]. In addition, the pH of the GPTMS aqueous solution at 0.1 - 10 vol% is around 4, which is the most stable region without requiring pH adjustment, so this property seems to be advantageous as a liquid component of paste.

In this chapter, HAp/Col-GPTMS pastes were prepared by mixing of HAp/Col powder and GPTMS aqueous solution, and the influences of the GPTMS concentrations and the powder to liquid (P/L) ratios on the physical properties of the pastes were investigated.

4. 1. Materials and methods

4. 1. 1. Preparation of HAp/Col-GPTMS paste

The powder phase of the HAp/Col pastes, HAp/Col powder, was prepared according to the 2.1.1.

HAp/Col powder, a powder phase of the HAp/ Col-GPTMS paste, was prepared by the following procedures: The as prepared HAp/Col was entered into a specially designed mold to allow squeezing of water during compaction and compacted by uniaxial pressing at 20 MPa. The HAp/Col compact was then freeze-dried, crushed, and classified to 100 μm or less in particle size by sieving. The liquid phase of the paste was GPTMS (Tokyo Chemical Industry Co., Ltd., Japan) aqueous solution just 1 h after mixing of GPTMS with distilled water (distilled water for injection, Otsuka

Pharmaceutical Co., Tokushima, Japan) to promote hydrolysis of GPTMS. Since the silane coupling agent is usually used at a concentration of 1 to 2 % in volume, the concentrations of the GPTMS solutions were 0.1, 1.0, and 10 % by volume. Pastes were prepared by mixing the GPTMS aqueous solution with the powder at several P/L ratios. The mixture conditions are summarized in Table 4-1. The pastes prepared with the respective concentrations of GPTMS solution are abbreviated as G[GPTMS concentration]-paste, e.g., the HAp/ Col paste prepared with 0.1 % in volume GPTMS solution is denoted as "G0.1-paste."

4. 1. 2. Decay property test

The washout property of the paste was measured in the same way as 2.1.4.

4. 1. 3. Viscosity test

The paste viscosity was measured according to 2.1.2 and 3.1.2.

4. 1. 4. Hardening behavior test

The HAp/Col-GPTMS pastes were a type of silicone hydrogel and demonstrated viscoelasticity; the a hardening behavior test using the Gillmore needle described in JIS T 0330–4 could therefore not be applied. Thus, the hardening behavior of the pastes was evaluated by the time-dependent spread area measurement described in 4. 1. 3. section. at 0, 10, 30, 60, 180, 360 and 1440 min additional aging before compression in the viscosity measurement; i.e., the result at 0 min indicates exactly the same condition as the viscosity test.

4. 1. 5. Compressive strength test

The compressive strength of the paste was measured according to the procedure described in JIS T 0330–4. The raw materials were mixed for 3 min and molded in a Teflon® mold to form a cylindrical shape 6.0 mm in diameter and 12.0 mm in height. They were then incubated at 37 °C, 95 ± 5% RH for 1 h in an incubator and soaked in 37 °C distilled water for 72 h. After soaking, the paste was removed from the mold, and its compressive strength was measured with a universal testing machine (AGS 5kN, Shimadzu, Japan) at a cross-head speed of 0.5 mm/min. The Young's

Table 4-1. Amount of liquid relative to raw material powder and P/L ratios.

Amount of powder / g	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Amount of liquid / cm^3	5.00	3.00	2.00	1.33	1.00	0.80	0.67	0.50
P/L ratio (g/cm^3)	0.20	0.33	0.50	0.75	1.00	1.25	1.50	2.00

modulus of the paste was calculated from the inclination of the elastic region in the stress-strain curve.

4.1.6. Statistical analysis

The results are shown as the mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance with the Tukey-HSD post hoc test. The statistical significance was set at $p < 0.05$.

4.2. Results

4.2.1. Preparation of the HAp/Col pastes

The apparent fluidities and viscosities of the HAp/ Col-GPTMS pastes just after mixing seemed to depend on their P/L ratios, regardless of the GPTMS concentration. The paste prepared at a P/L ratio of 0.20 shown in Figure 4-1(a) was a highly fluidic suspension, and that prepared at the P/L ratio of 2.00 shown in Figure 4-1(b) was aggregated. Since they could not be formed into monolithic cylinders within the stated period by the standard method, these pastes were excluded from further tests, i.e. the results include the pastes with P/L ratios between 0.33 and 1.50 that showed good kneading performance. Moreover, the paste with P/L ratio of 0.33 could be injected via 18 G needle (c) and the paste with P/L ratios between 0.50 and 1.50 could be injected via syringe with inner diameter 1.8 mm (d).

4.2.2. Washout properties

The washout ratios of the pastes are summarized in Fig. 4-2. All the pastes except G10-paste at a P/L ratio of 1.50 demonstrated sufficiently low washout ratios of less than 1 % mass. The G0.1- and G1.0- pastes showed minimum anti-washout ratios with a P/L ratio of 1.00; the G10-paste showed no observable decay up to a P/L ratio of 1.0, however, but, rather increasing its washout ratios with increases in the P/L ratio to 1.25 and 1.50.

4.2.3. Viscosity and hardening properties

Figure 4-3 shows viscosities of the paste represented as spread areas with have negative correlations to each other. Although the G10-paste showed the highest viscosity trend at each P/L ratio, the differences among different GPTMS

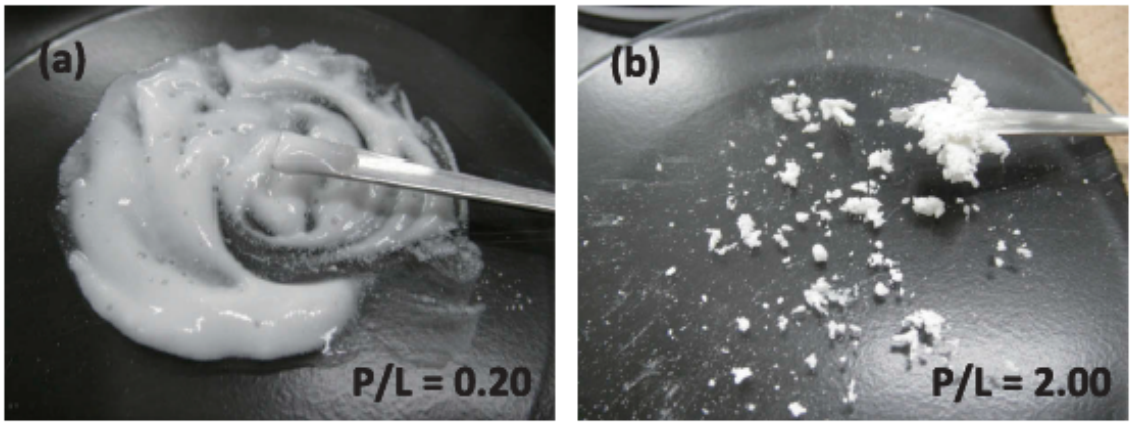

Figure 4-1. Appearance of the paste just after kneading: (a) P/L = 0.20, (b) P/L = 2.00.

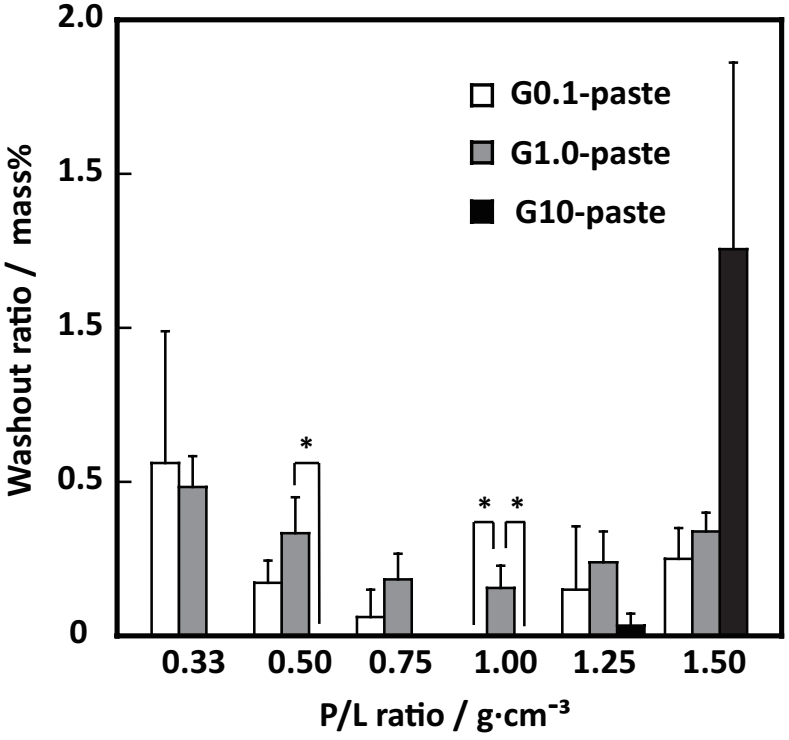

Figure 4-2. Static washout ratio of the pastes in PBS.

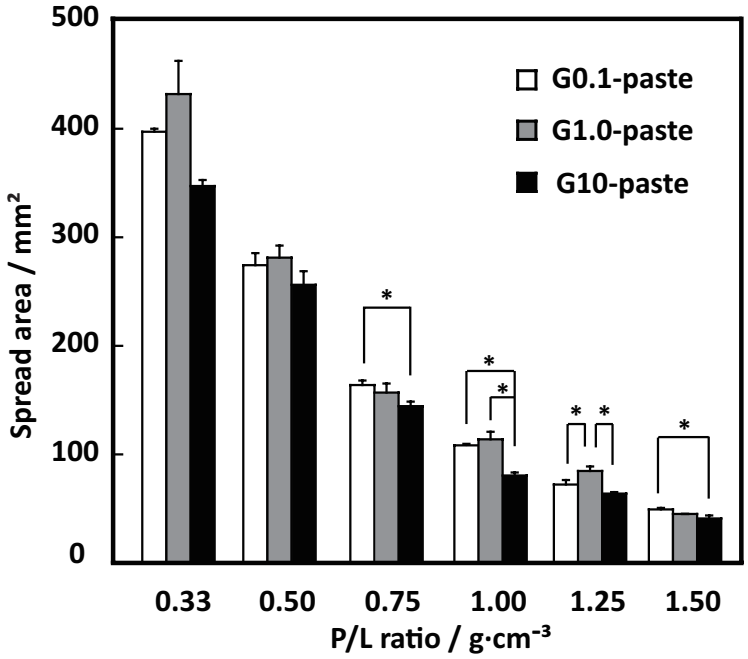

Figure 4-3. Spread area of 100 mm^3 of paste compressed by a 2 kg weight.

concentrations were not especially large. The viscosity of the pastes therefore depended hardening on the P/L ratios, even through some significant differences were observed among the different GPTMS concentrations, as similar to apparent one. Water was observed to be oozing from the pastes prepared at P/L ratios of 0.33 to 0.75 after the viscosity test, moreover, but not for the pastes prepared at P/L ratios of 1.00 to 1.50.

The hardening behaviors of the pastes are shown in Fig. 4-4. The pastes increased in hardness rapidly in the first 30 min and hardened gradually during the next 24 h. The relative increments of the pastes' viscosities in the first 30 min was in negative correlation to their P/L ratios. The G0.1-pastes were still completely spread up until loading at 24 h after incubation, and the final viscosity depended on their P/L ratios. By contrast, all the G1.0- and G10- pastes showed viscoelastic deformation at 24 h after incubation and similar viscosity to each other.

4.2.4. Compressive strength

During the compressive strength test, the G0.1-pastes showed plastic deformation without showing any obvious yield point; thus, the results for the G0.1-pastes are ignored. The compressive strengths of the G1.0- and G10-pastes are shown in Fig. 4-5. While the G1.0-pastes showed positive relations between their compressive strengths and P/L ratios and maximum strength at a P/L ratio of 1.50, the G10-pastes showed maximum compressive strength at a P/L ratio of 1.00. The Young's moduli of the pastes calculated from the stress–strain curves of the compressive strength tests, shown in Fig. 4-6, increased with increases in their P/L ratios. The Young's moduli of the G10-pastes were higher at each P/L ratio than those of the G1.0-pastes.

4.3. Discussion

The hardening process of HAp/Col-GPTMS paste can be assumed to be as follows:

(1) A reaction between the epoxy group of GPTMS and the amino groups on collagen molecules in HAp/Col particles begins immediately after mixing to immobilize the GPTMS molecules on the HAp/Col powders. In the meantime, a pH increase with a slight dissolution of HAp in the HAp/Col particles allows polymerization of the GPTMS to start in a step-growth fashion. This condensation occurs in both the immobilized and free GPTMSs. At this time, the viscosity of the paste increases but still

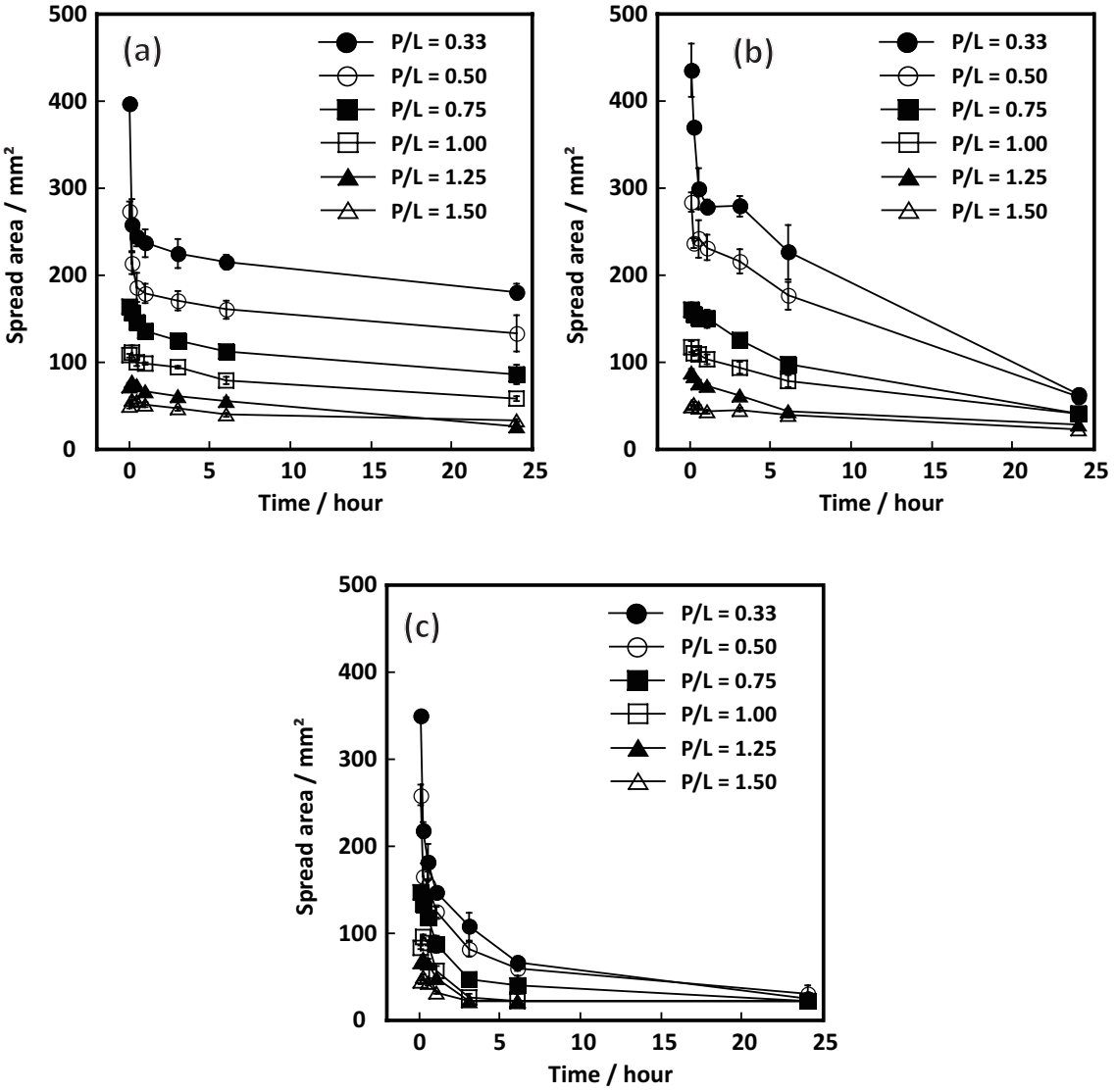

Figure 4-4. Time course of the pastes' viscosity: (a) G0.1-, (b) G1.0-, (c) G10-pastes.

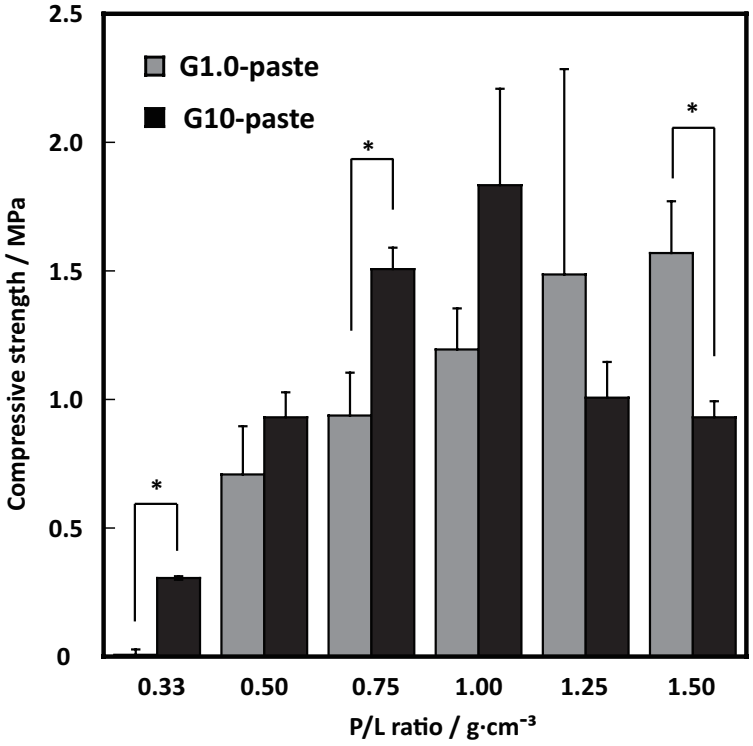

Figure 4-5. Compressive strength of the pastes other than the G0.1-pastes according to JIS.

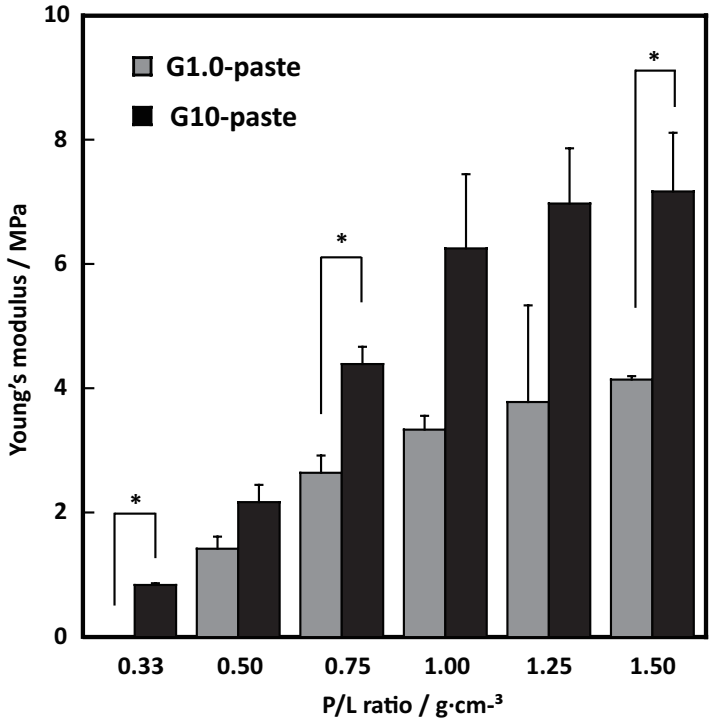

Figure 4-6. Young's modulus of the pastes calculated from the stress-strain curve of the compressive strength test, except for the G0.1-pastes.

retains its fluidity because the oligomers formed have only short molecular lengths.

(2) The GPTMS oligomers start to combine with each other as well as with GPTMS monomers. This process is theoretically slower than that in Step 1, but the paste's viscosity starts to decrease drastically. This step continues up to the near completion of large-scale network formation and attains a viscoelastic nature.

(3) The remaining silanol groups are incorporated into the large-scale network.

The hardening behavior test shows results based on the reaction process described above. The results of the hardening behavior test at 0 and 10 min after the start of mixing show the stage of shifting from Step 1 to Step 2. The rapid increase in viscosity up to 30 min after mixing indicates the rapid growth of the network described in Step 2. The viscosity 1 h after the start of mixing gently increases, and this is considered to be the state of Step 3.

The handling properties, determined mainly by the initial fluidity and formability of the HAp/Col- GPTMS paste just after mixing, were strongly influenced by the P/L ratios of the pastes as reported in conventional bone cements [9]. The relationship between the liquid volume of GPTMS for 1 g of HAp/Col powder and the spread area, shown in Fig. 4-7, revealed, moreover, that the spread area and the liquid volume were in a proportional relationship with a high correlation coefficient. The initial behavior of the paste is therefore independent of the immobilization of GPTMSs on the HAp/Col powder, but dependent on the powder-to-liquid ratio.

The paste prepared at a P/L ratio of 1.0 demonstrated the best anti-washout properties, and all other pastes tested in the present study showed even better anti-washout properties than commercially available bone cements [10].

Unlike the handling properties, the washout properties are influenced by the immobilization of GPTMS on the HAp/Col powder. The reasons are as follows:

(1) HAp/Col powders are easily dispersed from the paste mixtures in aqueous solutions, even in highly viscous solutions such as alginate solution [11].

(2) A preliminary anti-washout test for HAp/Col- GTMS pastes prepared with HAp/Col powder, in which collagen molecules were dehydrothermally crosslinked at amino and carboxyl groups, demonstrated rapid decay of the pastes with no retention of any GPTMS gels during the test period.

These results demonstrate that GPTMS immobilization via amino groups on collagen molecules in the HAp/Col powder is a key issue in the formation of a GPTMS

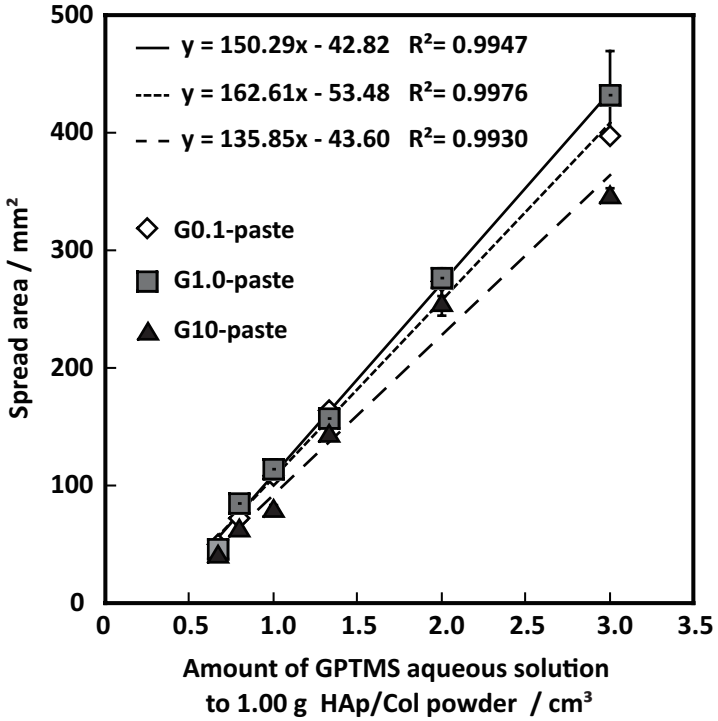

Figure 4-7. Correlation between the spread areas of the viscosity test and amount of the liquid phase of the pastes.

network (gel) under anti-washout test conditions, soaking of the paste in an aqueous solution. That is to say, increasing the viscosity by GPTMS oligomer formation is not sufficient to inhibit washout, as with alginate solutions. The influences of the P/L ratios on the anti-washout properties are also related to the hardening steps. Since almost the same volume of water absorption was observed for the HAp/Col powder in paste prepared at a P/L ratio of 2.0, absorption of water by HAp/Col powder has no effect on the formation of siloxane networks at high P/L ratios. The P/L ratios of 1.25 and 1.50 are approximately converted to the respective volume ratios of 0.6 and 0.75. Thus, the water remaining for free movement of GPTMS molecules is very limited. Accordingly, the probability of GPTMS immobilization and oligomer formation could be higher at the point of first contact between powder and liquid than at other points. As a result, some small aggregates were formed even when the paste seemed to be homogeneously mixed, and the aggregates decayed during the test. Thus, relatively homogeneous pastes prepared at lower P/L ratios have another issue with respect to anti-washout properties. Comparatively wide gaps between HAp/Col particles filled with GPTMS solution allow infiltration of the soaking liquid and inhibit siloxane network formation due to inhibition of siloxane bond formation by the dilution effect of the soaking liquid and widening of the gaps between particles.

The compressive strengths were obviously dependent on the GPTMS concentration. The compressive strengths of the G1.0-pastes increased with increases in the P/L ratio, because of the greater increase in the strength of the HAp/Col proportion in the paste than in the GPTMS gel; however, the compressive strengths of the G10-pastes showed the maximum value at a P/L ratio of 1.0 and decreased with increases in the P/L ratio for a reason similar to that of the anti-washout test, the paste homogeneity. The increasing density of the paste and decreasing homogeneity of the network in the paste competitively affect the compressive strength of the paste.

Young's modulus increased with increases in the P/ L ratio at any GPTMS concentration, due to an increase in the amount of HAp/Col powder. All the HAp/Col-GPTMS pastes showed a lower Young's modulus than that of conventional calcium phosphate bone cement [12, 13], but the low Young's modulus hydrogel would provide the HAp/Col-GPTMS pastes with a flexibility allowing deformation without fracture when they are inserted to fill in a closed part.

4.4. Summary

The pastes with P/L ratios between 0.33 and 1.50 showed good kneading performance and they could be injected via syringe with 1.8 mm inner diameter. Moreover, the pastes with P/L ratio of 0.33 could be injected via 18G needle.

The pastes using HAp/Col powder with non-dehydrothermal cross-link showed anti-washout property. Particularly, the G0.1- and G1.0- pastes showed minimum anti-washout ratios with a P/L ratio of 1.00.

The HAp/Col-GPTMS pastes after hardening showed lower young's moduli than conventional bone cements, and they demonstrated viscoelasticity.

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Chapter 5
Biological evaluation of hydroxyapatite/Collagen–(3-glycidoxypropyl)trimethoxysilane paste

The chapter 4 describes excellent physical properties as a bone void filler paste of the hydroxyapatite/collagen (HAp/Col)-(3-glycidoxypropyl)trimethoxysilane (GPTMS) paste. The HAp/Col itself is already confirmed its safety and excellent bone tissue reactions; however, biological properties including safety and bone tissue reactions of combination of the HAp/Col and GPTMS have not been investigated.

In this chapter, biological properties of the HAp/Col-GPTMS pastes were evaluated by cell culture tests and animal experiments using SD rats and a pig. In detail, initial resorption behavior of the paste was investigated by implantations of the paste into SD rats' tibia. In addition, long-term resorption was studied by a direct injection of the paste into pig tibia.

5.1. Materials and methods

5.1.1. Preparation of samples for biological test

Starting HAp/Col powder, without the dehydrothermal crosslink, and serial concentrations of GPTMS aqueous solutions were prepared according to the chapter 4 [1]. The pastes prepared with the respective concentrations of GPTMS solution are abbreviated as G[GPTMS concentration]-paste, *e.g.*, the HAp/ Col paste prepared with 1.0% GPTMS solution is denoted as "G1.0-paste."

The HAp/Col pastes for the biological tests described in the chapter were prepared from the HAp/Col powder sterilized with ethanol aqueous solution at 70% in volume and a filter sterilized GPTMS aqueous solution.

The HAp/Col-GPTMS pastes for a cytocompatibility test were prepared by mixing the powder and liquid portions at powder/liquid (P/L) ratios ranging from 0.33 to 1.50 (g/cm^3). The preparation conditions of the pastes for the cell culture test were chosen based on the results of mechanical property tests. The pastes were prepared on site and directly injected into a well as described in the following section. A HAp/Col dense body with the same size as the pastes, 5 mm in height and 7 mm in diameter, and a blank were used as controls. The 5-mm thick HAp/Col compact fabricated by the method described in 3.2.3. was punched out by a punch with an inner diameter of 2 mm

and was dehydrothermal cross-linked at 140 °C for 12 h. The obtained HAp/ Col dense body was then soaked in the Dulbecco's modified Eagle's medium (Sigma-Aldrich, UK; D-MEM) for 5 days to allow saturated adsorption of the Ca^{2+} and Mg^{2+} ions.

For an *in vivo* resorption behavior evaluation of the paste, G1.0- and G10-pastes at a P/L ratio of 1.00 were chosen because of their preferable conditions in both mechanical and handling properties. The HAp/Col-GPTMS mixture was filled into a hole prepared on a 2 mm thick silicone sheet by punching out using a punch with an inner diameter of 2 mm. The mixture in a hole aged for 3 day in an incubator at 37 °C and relative humidity of 100 % was used as an implanted specimen. A positive control (PC), the HAp/Col dense body with the same size as the paste specimen, was prepared by the same method mentioned above using the 2-mmΦ punch and 2-mm thick HAp/Col compact.

Specimens for the long-term bioresorbability of the HAp/Col-GPTMS pastes, G1.0- and G10-pastes in a P/L ratio of 1.00, chosen by the same reason for the *in vivo* resorption behavior test, were prepared on site, *i.e.,* implanted before complete hardening to simulate practical use.

5.1.2. *In vitro* cytocompatibility

A P/L ratio of 1.0 was chosen to investigate the influences of the GPTMS concentration on cytocompatibility, because G10-paste showed maximum compressive strength as well as complete anti-washout properties at this P/L ratio. All the G1.0-pastes were chosen for the cell culture test to investigate the influence of P/L ratios, due to the stable anti-washout properties at all the P/L ratios. A human osteoblastic cell line, MG-63, was used for the cytocompatibility test and all cell culture operations were performed at 37 °C in a 5% CO_2 humidified atmosphere. The D-MEM supplemented with 10% by volume fetal bovine serum (Cosmo Bio Co. Ltd., lot number 10D219; FBS) and 1% by volume penicillin and streptomycin (Invitrogen-Gibco; Pen/Strep) was used as the culture medium. A total of 2×10^4 cells were seeded in each well of a 6-well tissue culture polystyrene (TCPS) plate with the 2-cm^3 culture medium. One day after seeding, the culture medium was changed, and the paste, 5 min after the start of raw material mixing, was directly injected into each well through a syringe with an inner diameter of 7 mm to form the paste into a cylinder 5 mm in height and 7 mm in diameter. After that, the medium was changed every 2 days, and the number of cells

was counted at 1, 3, and 7 days after cell seeding with a hemocytometer. The silicon ion concentration of the medium collected at its change was measured with an inductively coupled plasma-atomic emission spectrometer (SPS7800, SII NanoTechnology, Japan; ICP-AES).

5.1.3. *In vivo* resorption behavior of the HAp/Col-GPTMS paste

Initial bone tissue reactions of the paste implanted in rat's tibiae were studied by histological observations. This animal test was approved by the Institutional Animal Care and Use Committee of National Institute for Materials Science (Permission number: 49-2016-3) and which was carried out according to the committee's guidelines.

The specimens prepared according to the section 5.1.1. were randomly implanted into bone holes measuring 2.0 mm in diameter prepared in the tibiae of SD rats (male, 8 weeks old) for 1, 2 and 4 weeks. Numbers of specimen for the experimental and the control groups were 4 and 3 for each period, respectively.

After the sacrifice of rats, the pastes and surrounding tissues were collected, observed with the naked-eye, and fixed with 10% formalin neutral buffer solution. The fixed specimens were analyzed X-ray micro-computed tomography (μ-CT; inspeXio SMX-90CT, shimadzu, Tokyo, Japan) of which the spatial resolution was approximately 0.019 mm/pixel at an X-ray voltage of 90 kV and 110 μA. The VGstudio MAX2.1 software (Volume Graphics GmbH, Japan) was used for volume measurement of specimen. The pastes portions in the μ-CT images were extracted as a region of interest and the volumes were measured and material reduction ratios were calculated from the result. In calculating the material reduction ratios, test pieces immersed in phosphate buffered saline for 1, 2 and 4 weeks were prepared as a standard to ignore the influence of swelling of HAp/Col. The material reduction ratios were calculated by the following formula.

$$\text{Material reduction ratio (\%)} = \frac{\text{Standard volume (mm}^3\text{)} - \text{Specimen volume (mm}^3\text{)}}{\text{Standard volume (mm}^3\text{)}} \times 100 \quad (5\text{-}1)$$

After the μ-CT observation, the specimens were decalcified for 2 weeks with 10% ethylenediaminetetraacetic acid disodium salts solution exchanged everyday. Standard dehydration in sequentially increasing ethanol solutions to 100% ethanol was performed followed by immersion in Histo-Clear®. Thereafter specimens were immersed in molten paraffin and embedded. Tissue blocks were sectioned at 5 μm and stained either by hematoxylin and eosin (HE) or tartrate-resistant acid phosphatase (TRAP), stained samples were observed with an optical microscope. Furthermore, lengths of the sample perimeter and that contacted with the TRAP activity marker were measured with ImageJ (NIH, ver. 1.46, for Mac). The TRAP activity coverage ratios were calculated from a following formula. Relations between the TRAP coverage ratio and material reduction ratio during observation periods were evaluated.

$$\text{TRAP coverage}\ (\%) = \frac{\text{Partial length with TRAP active}\ (-)}{\text{Perimeter of the residual samples}\ (-)} \times 100 \tag{5-2}$$

5.1.4. The long-term bioresorbability of the HAp/ Col-GPTMS pastes

The long-term bioresorbability of the HAp/Col-GPTMS pastes were evaluated by an animal test using a pig tibia, which was approved by the Institutional Animal Care and Use Committee of Meiji University (Permission number: IACUC15-0011) and which was carried out according to the committee's guidelines. The paste mixtures were packed into a cylindrical syringe of 4.0 mm in inner diameter to be a height of 8.0 mm and injected directly into 4 mm diameter bone hole prepared in the right tibia of a wild pig. At 12 weeks after the implantation, the pig was sacrificed and the implant site was harvested with the surrounding tissues. The pastes and surrounding tissues were observed with the naked-eye and by the μ-CT.

5.2. Result

5.2.1. Cytocompatibility test

5.2.1.1. Influences of GPTMS concentrations

Figure 5-1(a, b), respectively, show cell proliferation curves and Si ion concentrations in the culture medium during the cytocompatibility test. At day 3, cells cultured with the G0.1-paste proliferated as the same rate as the dense HAp/Col control,

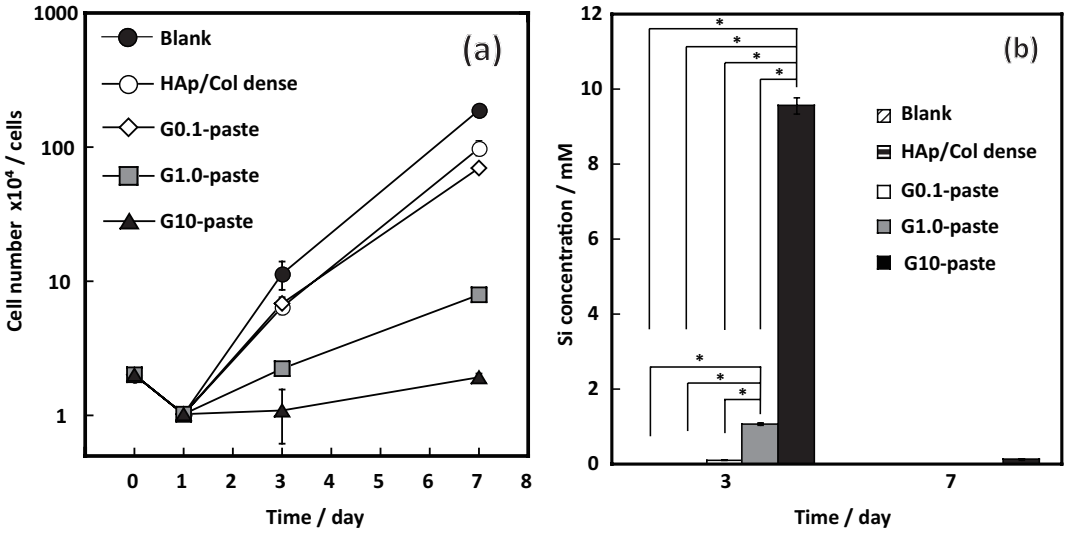

Figure 5-1. (a) Growth curve and (b) Si concentration of culture media cultured using P/L = 1.00 pastes.

whose safety is already confirmed by both animal tests and practical medical treatment of humans. The G10-paste strongly inhibited cell proliferation in the first 2 days, and the results for the G1.0-paste were in the middle range. Cell proliferation was positively correlated to the Si ion concentration at day 3, and the concentrations were obviously correlated to the GPTMS concentrations of pastes. At day 7, after the medium had been changed twice before Si ion measurement, Si ions were still detected in the medium in which the G10-paste was soaked at 0.124 ± 0.006 mM, while Si ions were no longer detected in the other media.

5.2.1.2. Influence of P/L ratios

As shown in Fig. 5-2(a), the cell numbers at day 3 showed the following order of P/L ratios: 1.50 ≈ 1.25 > 1.00 ≈ 0.75 ≈ 0.50 < 0.33. Although the order changed slightly for the P/L ratios of 0.50 to 1.00 at day 7, the cell number trend remained closely similar to that at day 3. A huge difference in cell numbers was observed between the P/L ratios of 1.00 and 1.25. Fig. 5-2(b) shows that the Si ion concentrations were the same for the P/L ratios from 0.33 to 1.00 but that they decreased with decreases in the liquid amounts of the G1.0-pastes at day 3. No Si ions were detected at day 7.

5.2.2. *In vivo* resorption behavior

5.2.2.1. μ-CT observation and material volume reduction

No irregular inflammation signs during the breeding and the harvested tissues were observed. The Images of μ-CT, shown in Fig. 5-3 showed that the pastes decreased in volume with increasing in time without density change. In contrast, the PC became smaller with the time lapse, and the material image became thin with a swelling of material.

The material reduction ratios were shown in Fig. 5-4. The PC volume could not be measured due to unclear boundary of the PC by swelling and resorption..The material reduction ratio increased with the passage of the implantation period for any GPTMS concentration. At the forth week, reduction ratio showed a significant difference between the G1.0- and G10-pastes. .

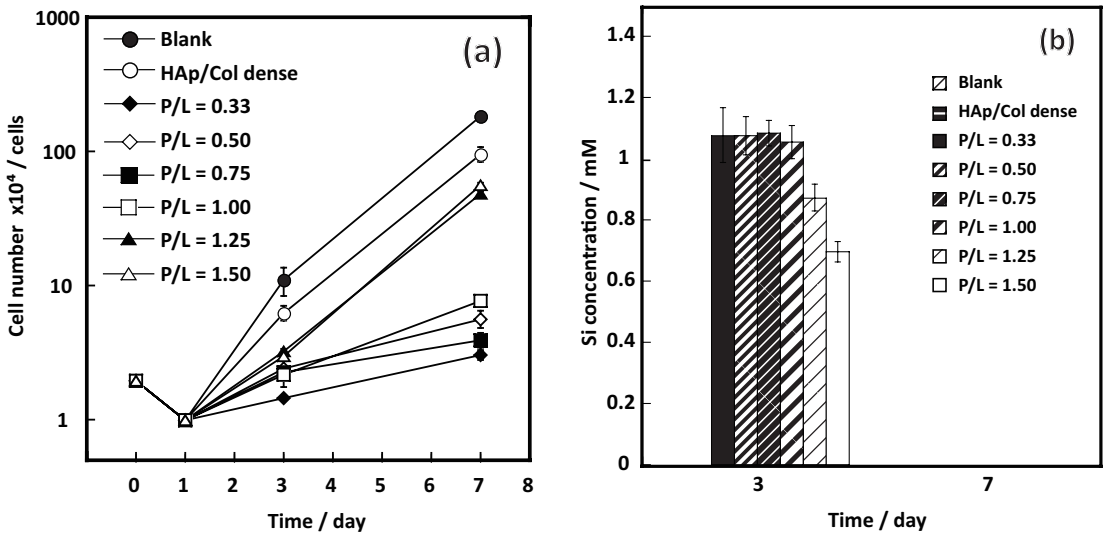

Figure 5-2. (a) Growth curve and (b) Si concentration of culture media cultured using G1.0-pastes.

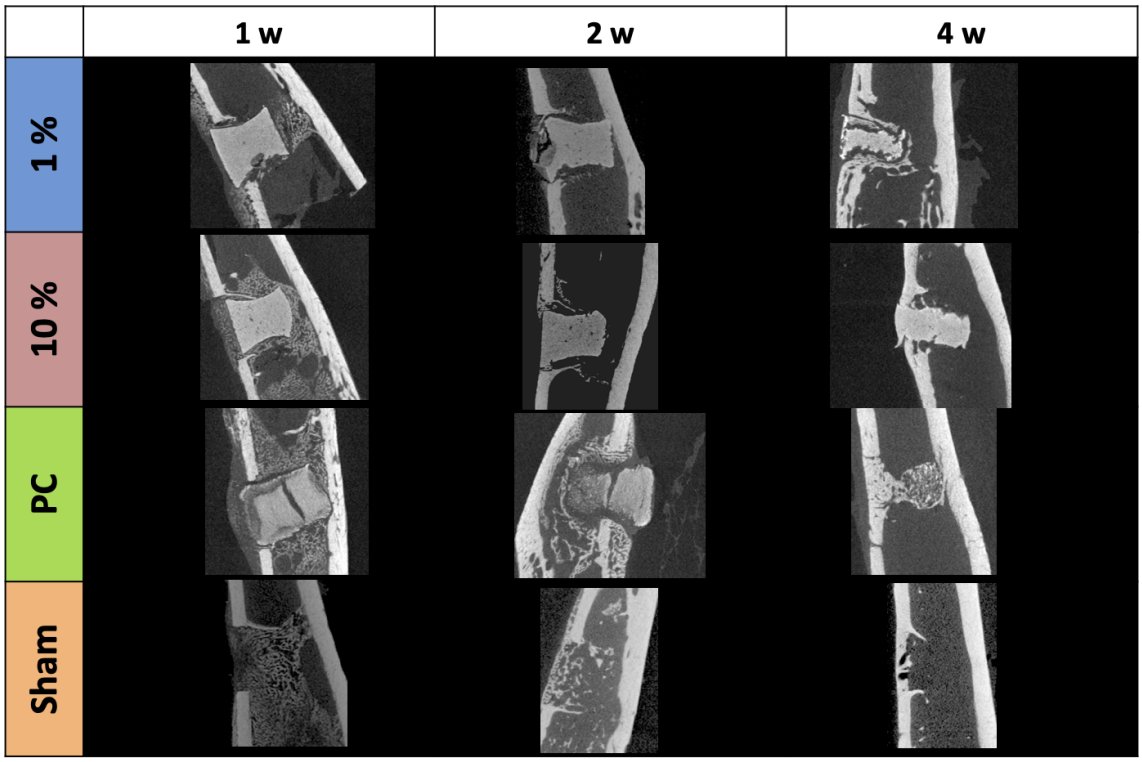

Figure 5-3. μ-CT images of the in vivo resorption behavior test at the time of harvesting.

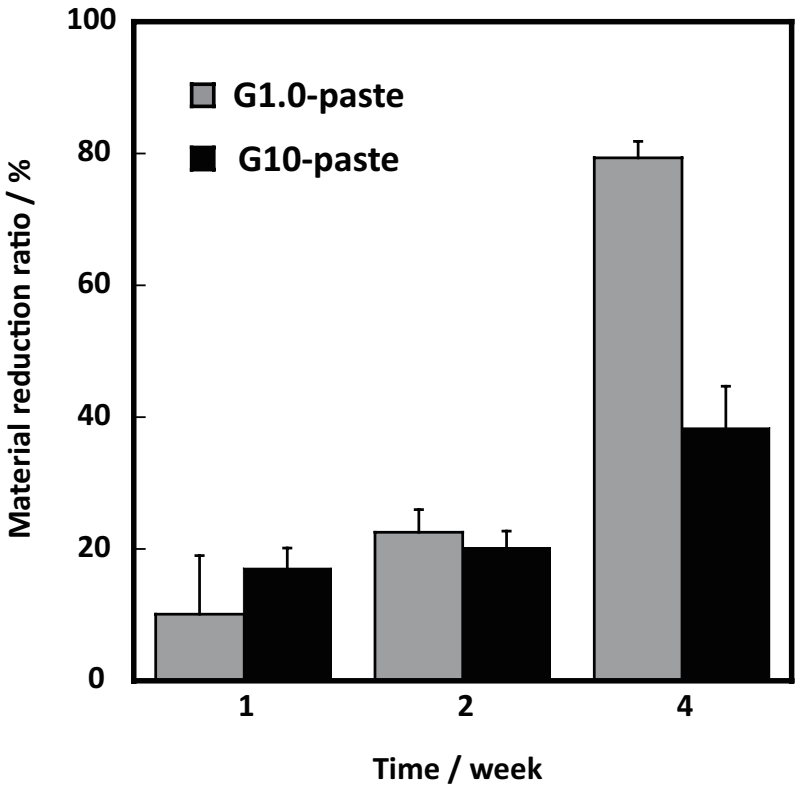

Figure 5-4. Material reduction ratio of the in vivo resorption behavior test.

5.2.2.2. Histological observation

The HE stained specimens are shown in Fig. 5-5. Osteogenic responses were observed around all implanted materials. The pastes seem homogeneous and dense, and cells were observed only on the peripheral of the pastes. Contrarily, lamellar structure and gaps between the lamellar was found for the PC. Cell penetration to the gap was observed.

The similar results were found at the second week. Some cracks formed on the G1.0-paste were observed at the forth week, and cells migrated into the cracks, whereas the G10-paste was still intact and no cell migrations were observed as the same as the previous weeks. Figure 5-6 demonstrates TRAP stained images. The TRAP active cells were observed on the pastes and PC from the first to fourth week with periodical increasing of an expression ratio of the TRAP active cells. In contrast, the TRAP active cells could not be observed with all sham controls during the test period.

The TRAP activity coverage ratios shown in Fig. 5-7 illustrated that the TRAP coverage ratio of the G1.0-paste became drastically increased with increasing in the implantation period; however, that of G10-paste showed small increment from the second week to fourth week. Relations between the material reduction ratio between each observation period and TRAP activity coverage ratio illustrated in Fig. 5-7.5 had linear relation with $R^2 = 0.94213$.

5.2.3. The long-term bioresorbability

The pig showed no systemic or local symptom during the test period. After its sacrifice, no obvious differences between original host bone and the paste implantation sites were observed by a naked-eye observation, *i.e.*, all the pastes seemed to be completely substituted with regenerated bone. Even in a μ-CT observation shown in Figure 5-8, the paste-implanted sites were not distinguished clearly. The μ-CT images also demonstrated complete substitution of the paste-implanted sites with newly formed bone as illustrated in the Fig. 5-8 as dotted-line circles, that was the estimated paste-implanted sites from photos taken at the implant surgery and from a faint contrast in the μ-CT images.

5.3. Discussion

Since the timing of immersion of the test samples in the solution in the

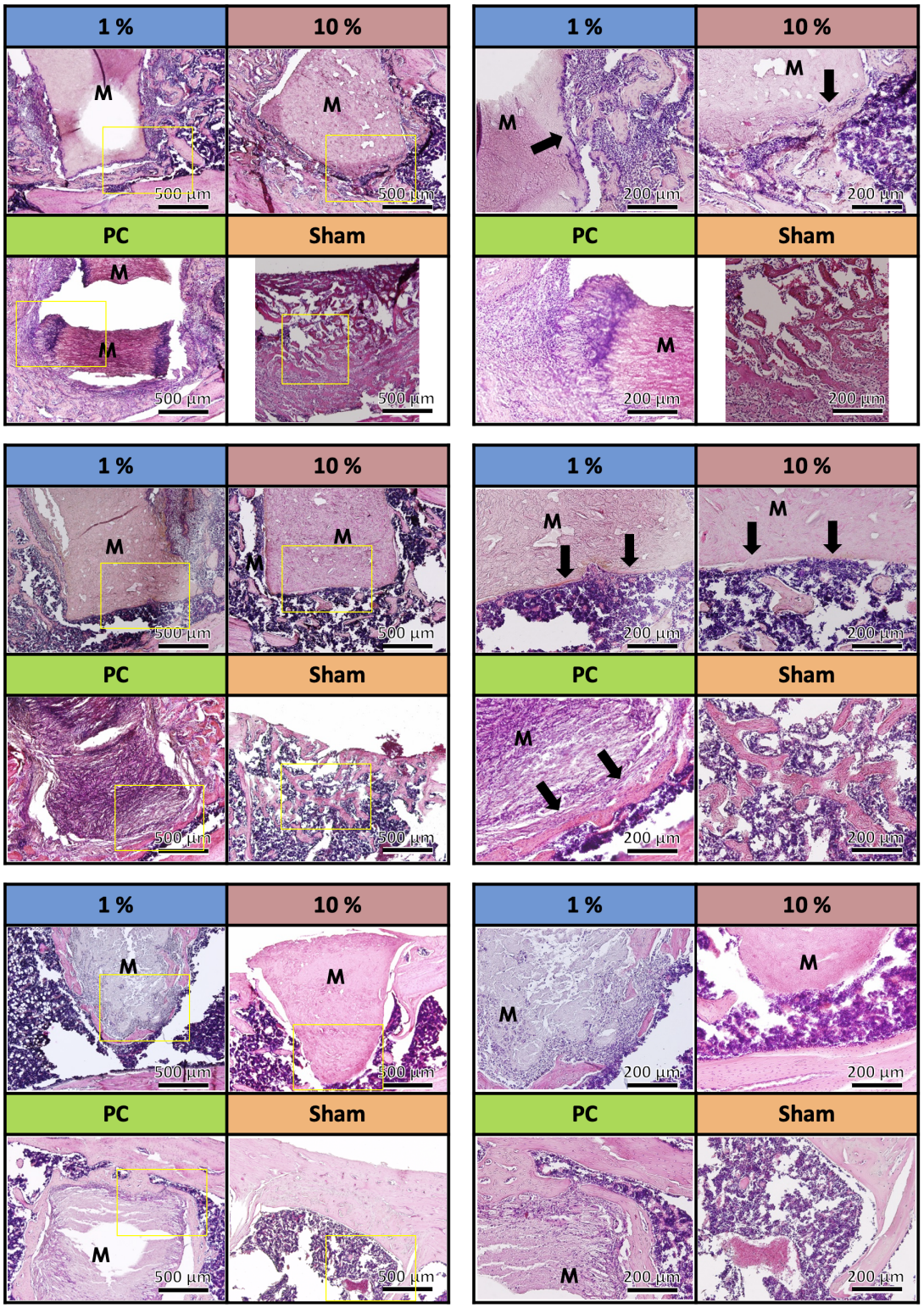

Figure 5-5. Histological observation of the HE stained specimens. (a)1 w, (b) 1 w with high magnification, (c) 2 week, (d) 2 w with high magnification (e) 4 w, (f) 4 w with high magnification.

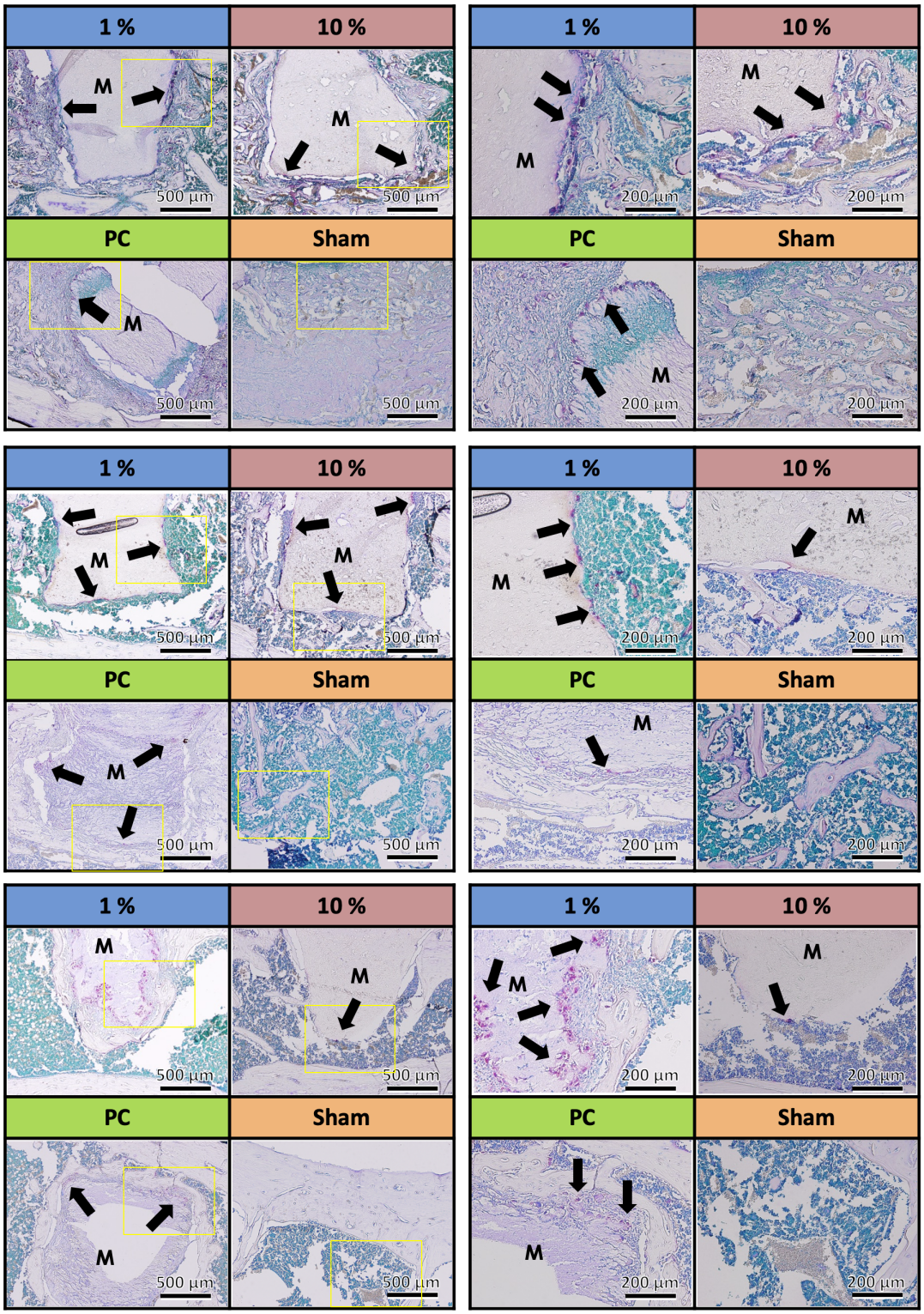

Figure 5-6. Histological observation of the TRAP stained specimens. (a)1 w, (b) 1 w with high magnification, (c) 2 week, (d) 2 w with high magnification (e) 4 w, (f) 4 w with high magnification.

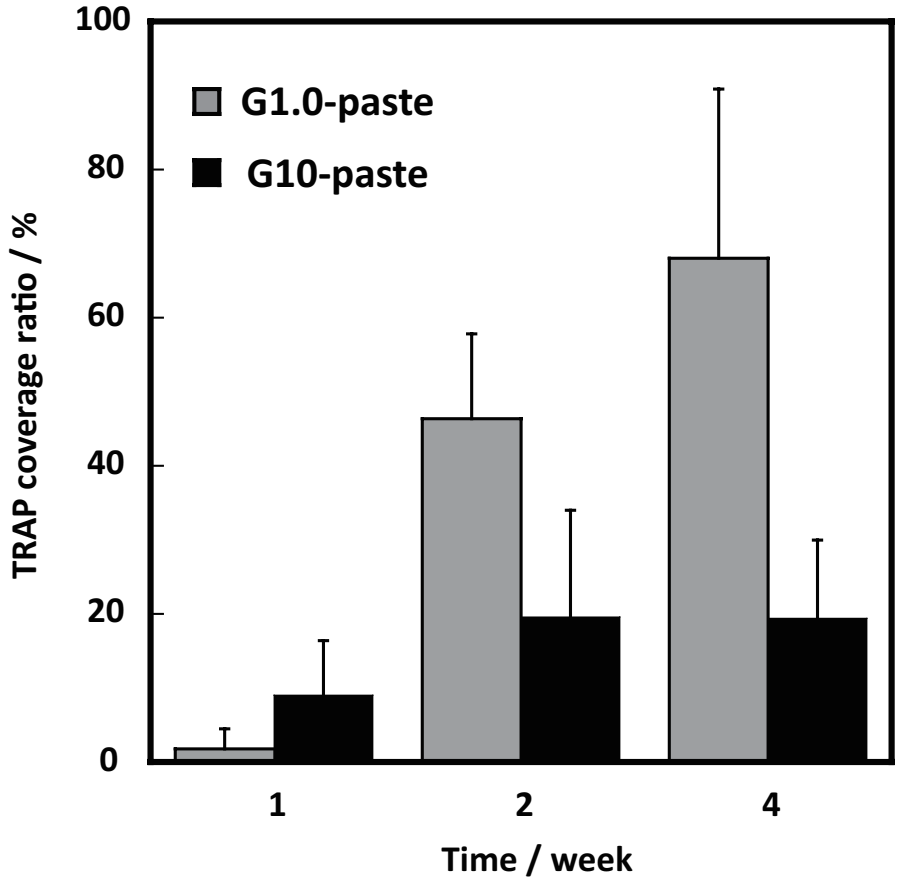

Figure 5-7. The TRAP activity coverage of the G1.0- and G10-pastes.

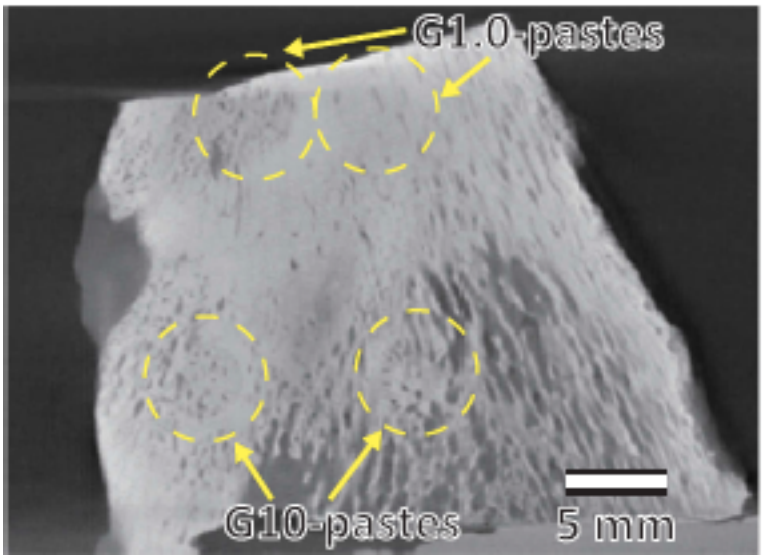

Figure 5-8. μ-CT image of G1.0- and G10-pastes implanted into a pig's tibia.

cytotoxicity test falls into the transition period from Step 1 to Step 2, the Si ion concentrations in the culture medium are interpreted to the amounts of the GPTMS leached from the paste during the setting reaction because no Si was contained in HAp and collagen. Thus, approximately 40.1% of GPTMS was eluted from G1.0-paste with a P/L ratio of 1.00, before the first medium exchange, but no Si ions were detected on day 7 in the medium, *i.e.* the rest of the GPTMS observed was immobilized in the paste. In the cytotoxicity test, the cytostatic effect was found from day 1 to day 3, *i.e.*, until the first medium change. Hence, the eluted GPTMS showed negative influence on the cell proliferation, because it may attack to cell surface directly and/or may react with important chemicals, including proteins in the medium, to reduce cell proliferation. A threshold for a drastic reduction in cell proliferation under the present culture conditions could exist between the P/L ratios of 1.00 and 1.25 for the G1.0-pastes. The half lethal concentration of GPTMS to MG63 reported by Shirosaki *et al.* was almost equal to the GPTMS concentration in the medium of G1.0-paste with P / L ratio of 1.00, and it supported the results this time [2]. The sufficiently low GPTMS amount showed a few reduction effects on cell growth, as seen in cells cultured with the G10-paste at day 7. In addition, a conventional cell culture only uses a very small amount of medium and effects of leaching and/or adsorption of substances sometimes translated with some exaggeration in comparison to *in vivo* reactions that has a large amount of body fluid flows. Therefore, the effects of the eluted GPTMS may be limited in *in vivo* conditions. In fact, any animal tests in this chapter demonstrated no harmful symptoms during the test period.

Supporting to the cytocompatibility test, no toxic signs were observed in this animal study. In the μ-CT observation until one-month, the paste after hardening decreased the volume without changing its density, instead the PC decreased its density. This was caused by difference swelling behavior between the PC and pastes resulting from their preparation methods. The PC had the lamellar structure and interfiber swelling was easier than homogeneous hydrogel-like structure of the pastes. The absorption rates of the pastes depended on the concentration of the GPTMS because of differences in the cross-linking density. The similar results on the difference in absorption rate depending on crosslinking density was reported for glutaraldehyde cross-linked HAp/Col dense body [3]

A gap formation on the PC is due to the fabrication method, the HAp/Col

fibers stacked perpendicular to compressive load, accordingly cells were only penetrated to the PC at the first week. On the other hand, the pastes were the mixture of the HAp/Col particles and the GPTMS solutions; therefore, their structure is homogeneous, thus, isotopic swelling was occurred without formation of gaps. However, with progress of resorption, cracks were formed on the G1.0-paste and cell migration was observed at the fourth week, whereas no crack formation nor cell penetration were observed for G10-paste. These differences were explained by cross-linking density relating to a resorption rate due to differences in GPTMS concentrations.

Presence of TRAP-positive multinucleated giant cells in the surface of the pastes and time-dependent reduction of the pastes indicated that the pastes were resorted by osteoclasts as the same as the PC. With the following bone formation observed histologically, the pastes were incorporated into bone remodeling process as the same as the HAp/Col reported previously.

Good linear relation between the material reduction ratio and the TRAP activity coverage ratio, trial for digitizing TRAP activity, roughly validated correctness of the digitized value, because the HAp/Col does not dissolve in the body fluid, which is supersaturated to HAp, and does only decomposed mainly by osteoclastic resorption (Fig. 5-9). Further validations are necessary to use the index generally, however, it can be useful for estimation of the HAp/Col reduction only by observation of TRAP stained sections.

Complete bone regeneration observed in the long-term bioresorbability of the HAp/Col-GPTMS pastes demonstrated that the pastes were complete bioresorbable with a new bone formation. This result and initial bone tissue reaction as described above, the pastes were completely incorporated into bone remodeling process, as the same as previous reports. Therefore, the HAp/Col-GPTMS pastes with appropriate GPTMS concentrations are good candidates for bioresorbable bone paste.

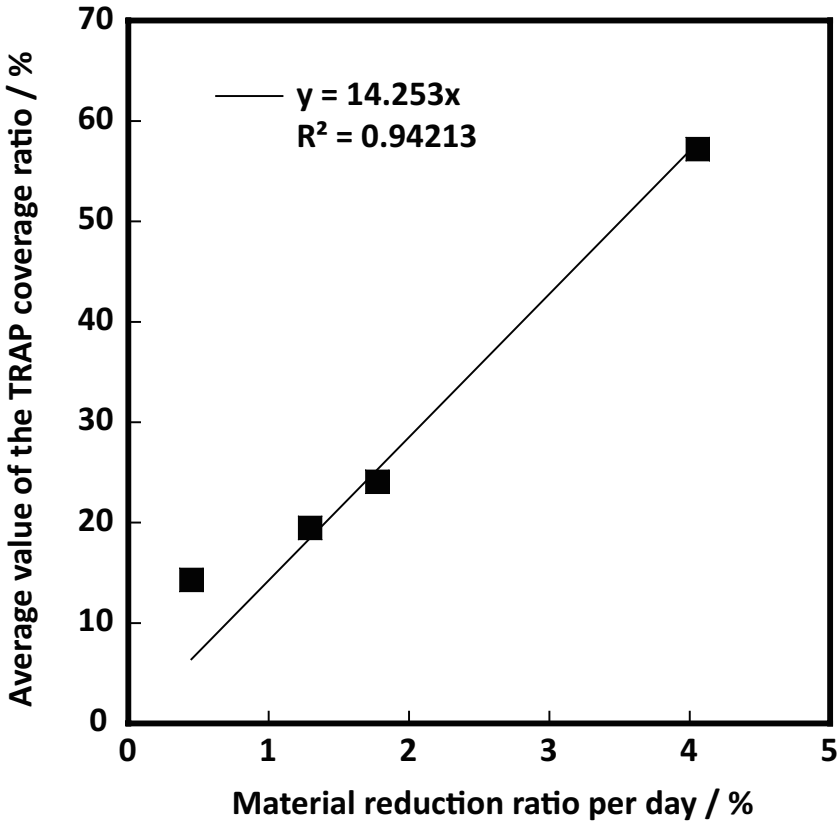

Figure 5-9. The material reduction ratio per day in each period and the TRAP coverage ratio in each period.

5.4. Summary

Although increasing the amount of GPTMS inhibited cell proliferation in a cell culture environment, no systemic or local symptoms except for the local inflammation generally observed after surgery were observed during the implantation test. Furthermore, the pastes were resolved by osteoclasts, and were completely substituted by newly formed bone via bone remodeling process. Hence, HAp/Col-GPTMS pastes are good candidates for use as a novel paste-type bioresorbable bone void filler.

References of chapter 5

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[3] M. Kikuchi, H. N. Matsumoto, T. Yamada, Y. Koyama, K. Takakuda, J. Tanaka, "Glutaraldehyde cross-linked hydroxyapatite/collagen self-organized nanocomposites", *Biomaterials*, **25**, 63-69 (2004).

Chapter 6 Conclusion

Calcium phosphate cements, of which the main component after setting is hydroxyapatite (HAp), have been widely used due to their biocompatibility, injectability and ability to fit irregularly shaped bone defects as well as these self-setting ability. Since the rather low resorption rate of HAp causes a decrease in the new bone formation rate; however, injectable bone void fillers composed mainly of higher bioresorbable materials are being prepared to achieve acceleration in bone formation. The hydroxyapatite/collagen bonelike nanocomposite (HAp/Col) proposed by Kikuchi has a bone-like nanostructure, and it is the first material that is completely incorporated into the bone remodeling process and whose resorption rate can be controlled by the crosslink ratio of its collagen content. Sponge-like porous HAp/Col, sold as Refit® in Japan. Hence, HAp/Col is a promising candidate for use as a bioresorbale bone substitute for other materials besides porous materials.

The purpose of this study was to prepare a self-setting injectable HAp/Col paste. For that purpose, preparation of raw materials, kind of hardening agent, raw material mixing ratio and addition of hardening reaction accelerator were studied.

In chapter 1 provided general information of HAp/Col, sodium alginate (Na-Alg), (3-glycidoxypropyl)trimethoxysilane (GPTMS) and aims of the present study.

In chapter 2, the suitable mixing conditions of the paste were a P/L ratio of 0.6 at the 90:10 mass ratio of Ca-HAp/Col and solution of low viscous Na-Alg. All additives increased the paste viscosity; however, the mechanisms were different between organic acids and calcium compounds. Organic acids rapidly decreased pH to form Alg gel by deposition of Alg. Contrarily, calcium compounds supplied Ca^{2+} ions to form egg-box structure for gelation of Alg, and the reaction depended on solubility of compound. Additives also increased decaying time but could not realize anti-decay in the present conditions.

In chapter 3, the anti-washout property, viscosity, and cytocompatibility to an osteoblastic cell line, MG-63, of anti-washout pastes were investigated. Mixing the HAp/Col, an aqueous solution of Na-Alg, which is a paste hardening and lubricant agent, and supplementation of calcium carbonate or calcium citrate (Ca-Cit) as a calcium resource for the hardening reaction realized an injectable bone paste. Adding

Ca-Cit at a concentration greater than eight times the Ca^{2+} ion concentration to Na-Alg improved the anti-washout property. Although the viscosity test indicated a gradual increase in the paste viscosity as the calcium compounds increased, pastes with excess supplementation of calcium compounds exhibited injectability through a syringe with a 1.8 mm inner diameter, realizing injectable bone filler. Furthermore, the anti-washout pastes with Ca-Cit had almost the same cell proliferation rate as that of the HAp/Col dense body.

Chapter 4 described preparation of anti-decay self-setting pastes of HAp/Col utilizing GPTMS. The powder portion of the paste was ball-milled HAp/Col synthesized by the simultaneous titration method, and the liquid portion was GPTMS aqueous solution at a concentration of 0.1, 1.0 or 10% in volume. The HAp/Col-GPTMS pastes were prepared by mixing the powder and liquid portions at powder/liquid (P/L) ratios ranging from 0.20 to 2.00 (g/cm^3). The pastes with P/L ratios from 0.33 to 1.50 showed good handling properties, and their viscosities depended greatly on the P/L ratio. The lowest washout ratio was observed at a P/L ratio of 1.00 independent of the GPTMS concentration.

In chapter 5, although cytocompatibilty tests showed that inhibition of cell proliferation depended on the elution amounts of GPTMS from the pastes, an animal test using porcine and rat tibia demonstrated no harmful systemic or local symptoms, because the GPTMS concentration maintained acceptable levels for living tissues through dispersion with body fluid. The animal test also revealed that the pastes were completely resorbed and substituted with newly formed bone within 12 weeks implantation.

This thesis concluded based on these results that HAp/Col pastes are promising candidates for use as bioresorbable injectable pastes.

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15th January 2019

Taira SATO