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FULL LENGTH ARTICLE

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

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ABSTRACT

This article describes preparation of anti-decay self-setting pastes of hydroxyapatite/collagen (HAp/Col) utilizing (3-glycidoxypropyl)trimethoxysilane (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. Although cytocompatibilty tests showed that inhibition of cell proliferation depended on the elution amounts of GPTMS from the pastes, an animal test using porcine 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 paste was completely resorbed and substituted with newly formed bone after 12 weeks implantation. It was concluded based on these results that HAp/Col-GPTMS pastes are promising candidates for use as bioresorbable injectable pastes.

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Hydroxyapatite/collagen nanocomposite; (3-glycidoxypropyl) trimethoxysilane; bone paste; mechanical properties; biocompatibility

1. Introduction

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 [1–3], injectable bone void fillers composed mainly of higher bioresorbable materials are being prepared to achieve acceleration in bone formation. Konishi et al. proposed chelate-setting calcium phosphate cements utilizing inositol hexaphosphate as a chelating agent and prepared self-setting cements composed of α- or β-tricalcium phosphates (β-TCP) as well as HAp cements. These cements indicated good biocompatibility, and α- and β-TCP cements were partly resorbed during 4 weeks observation and showed new bone formation in their bioresorbed areas [4,5]. The inositol hexaphosphate in the β-TCP cement needs many biological tests to verify its use as a setting agent, however, because it has not yet been tested for any physiological reactions, including toxicity as a substance directly applied in internal tissues, even if it is used as an oral supplement. The bioresorption mechanism of β-TCP is still unclear [6], moreover, and a clinical study reported that no osteoclastic resorption of a large quantity of β-TCP was observed 72 weeks after grafting [7], even though porous β-TCP ceramics are used worldwide as bioactive and biodegradable bone void fillers [8].

Since bone is a nanocomposite of HAp and type-I collagen, injectable bone void fillers composed of apatitic calcium phosphate and type-I atelocollagen have been sold worldwide as Boneject® (Koken, Japan) and Collagraft® (NeuColl, USA) [9–11]. They are simple mixtures of these contents; thus, their bone tissue reactions are phagocytosis of collagen by macrophages and osteoconduction to calcium phosphate particles with slight resorption by osteoclasts. In addition, they require a thick tube to inject them into bone defects. The hydroxyapatite/collagen bone-like nanocomposite (HAp/Col) proposed by the authors [12–15] has a bone-like

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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, demonstrated faster bone regeneration and filler resorption than those of porous β-TCP in clinical trials, moreover, and is clinically used in Japan [15]. Hence, HAp/Col is a promising candidate for use as a bioresorbale bone substitute for other materials besides porous materials. We recently reported on HAp/Col pastes using sodium alginate as a hardening agent [16–18] and concluded that supplementation of large amounts of calcium salts other than HAp was necessary to obtain sufficient anti-washout properties. Even though the chemicals calcium citrate and calcium carbonate show no harm to tissues, huge amounts of them might decrease and/or inhibit the biological advantages of HAp/Col.

To solve this problem, silane coupling agents were proposed as a new hardening agent for HAp/Col paste. Silane coupling agents are commonly reported as functional materials to prepare biomaterials composed of inorganic or organic materials or their composites [19–21], because silane coupling agents generally have the property of generating silanol groups by hydrolysis, and because they bond covalently to inorganic substances and also form siloxane networks by their self-condensation [22]. In this study, (3-glycidoxypropyl)trimethoxysilane (GPTMS) had been chosen because epoxy groups in GPTMS bond to amino groups in collagen [23–26].

In this paper, 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. The biocompatibility of the HAp/Col-GPTMS pastes was evaluated, moreover, by the conventional cell culture test and an animal test using pig tibia.

2. Materials and methods

2.1. Materials

HAp/Col was synthesized at a HAp to collagen mass ratio of 80:20 by the simultaneous titration method [12,13]. Briefly stated, 10 g of HAp/Col were synthesized from 205.0 cm^3 of 400 mM $Ca(OH)_2$ suspension, which was prepared by hydration of calcium oxide obtained by burning calcium carbonate ($CaCO_3$, Alkaline analysis grade, Wako Pure Chemicals Inc., Japan) at 1050 °C for 3 h and 404.9 cm^3 of 120 mM orthophosphoric acid aqueous solution (Reagent grade, Wako Pure Chemicals Inc., Japan) with 2.0 g of type-I porcine dermal atelocollagen (Biomaterial grade, Nitta Gelatin Inc., Japan). They were simultaneously titrated into a reaction vessel containing 199.1 cm^3 of distilled water at a titration rate of 30 cm^3/min. The temperature and pH of the reaction solution were 37 °C and 9, respectively.

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. 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 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 % GPTMS solution is denoted as "G0.1-paste."

2.2. Washout property test

The washout properties of the paste was evaluated according to the procedure described in Japanese industrial standard JIS T 0330–4 "Bioceramics-Part 4: Physicochemical characterization of calcium phosphate bone paste." In detail, a paste mixture at 3 min after the start of mixing was packed into a syringe 4.8 mm in inner diameter and 16.5 mm in height, and the packed paste was squeezed onto a wire net with a wire diameter of 0.5 mm and aperture of 2.0 mm. At 5 min after the start of mixing, the paste and wire net were soaked in 50 cm^3 of Dulbecco's phosphate buffered saline (PBS) at 37 °C in an appropriate container. They were then placed statically in an incubator at 37 °C, 95 ± 5 % relative humidity (RH) for 72 h. The washout ratio was calculated as the mass percentage of the paste debris on and beneath the wire net with respect to the original mass of the paste.

2.3. Viscosity test

A viscosity of the paste was measured according to Ishikawa's method [27]. Briefly, the paste mixture at

Table 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

exactly 3 min after the start of mixing was shaped into a cylinder at a 5.0 mm in diameter and 5.1 mm in height, of which the volume was approximately 0.1 cm^3. The paste cylinder was placed on a glass plate and spread at exactly 10 min after the start of mixing by compression with a glass plate and a total weight of 2 kg in mass. After 10 min spreading, the spread area of the paste was measured using its digital image with ImageJ (NIH, ver. 1.46, for Mac) to determine its viscosity.

2.4. 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 2.3 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.

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

2.6. Cytocompatibility test

HAp/Col paste for the cytocompatibility test was prepared from the HAp/Col powder sterilized with ethanol aqueous solution at 70% in volume and a filter-sterilized GPTMS aqueous solution. 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 Dulbecco's modified Eagle's medium (Sigma-Aldrich, UK; D-MEM) supplemented with 10 % by volume fetal bovine serum (FBS, Cosmo Bio Co. Ltd., lot number 10D219) and 1 % by volume penicillin and streptomycin (Invitrogen-Gibco; Pen/Strep) was used as the culture medium. A total of 20,000 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. 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). A HAp/Col dense body with the same size as the paste specimen was prepared by the compacting method for the HAp/Col described in 2.1 and by die cutting with a punch, followed by dehydrothermal cross-linking at 140 °C for 12 h and soaking in the D-MEM for 5 days to allow saturated adsorption of the Ca^{2+} and Mg^{2+} ions. The preparation conditions of the pastes for the cell culture test were chosen based on the results of mechanical property tests. The HAp/Col dense body and a blank were used as controls.

2.7. Animal test

The biocompatibility and bioresorbability of the HAp/Col-GPTMS pastes were preliminarily evaluated by an animal test, which is 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. G1.0- and G10-pastes in a P/L ratio of 1.00 were chosen for the animal test and prepared on site, i.e. implanted before complete hardening to simulate practical use. The paste mixtures were packed into a cylindrical syringe 4.0 mm in inner diameter to a height of 8.0 mm and injected directly into bone holes measuring 4 mm in diameter prepared in the right tibia of a wild pig. At 12 weeks after 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 micro X-ray micro-computed tomograph (μ-CT).

2.8. 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 $p < 0.05$.

3. Results

3.1. 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 1(a) was a highly fluidic suspension, and that prepared at the P/L ratio of 2.00 shown in Figure 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.

3.2. Washout properties

The washout ratios of the pastes are summarized in Figure 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.

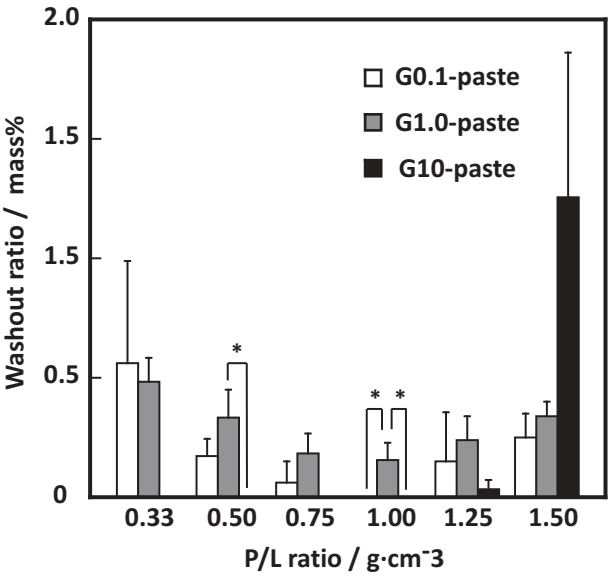

Figure 2. Static washout ratio of the pastes in PBS.

3.3. Viscosity and hardening properties

Figure 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 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.

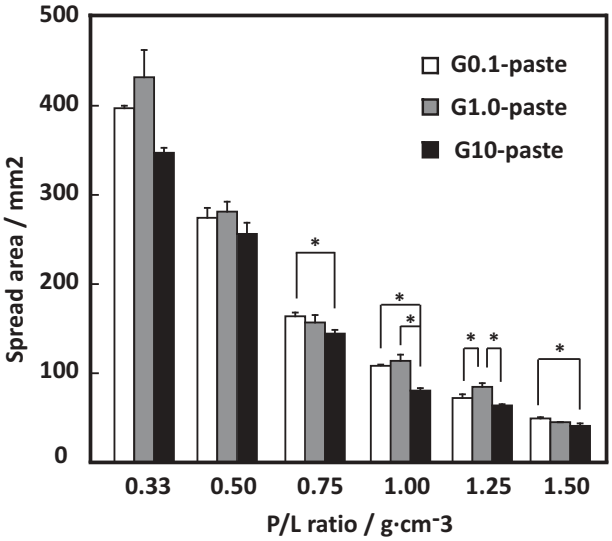

Figure 3. Spread area of 100 mm^3 of paste compressed by a 2 kg weight.

The hardening behaviors of the pastes are shown in Figure 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.

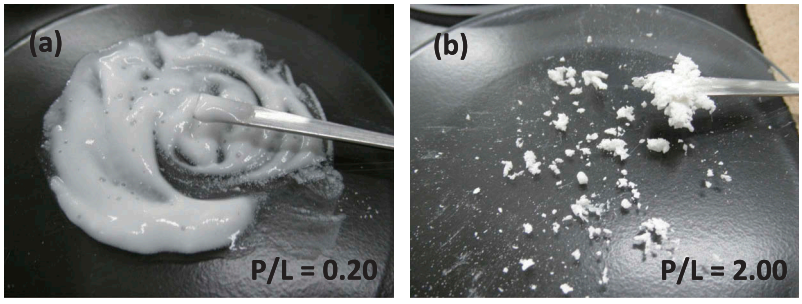

Figure 1. Appearance of the paste just after kneading: (a) P/L = 0.20, (b) P/L = 2.00.

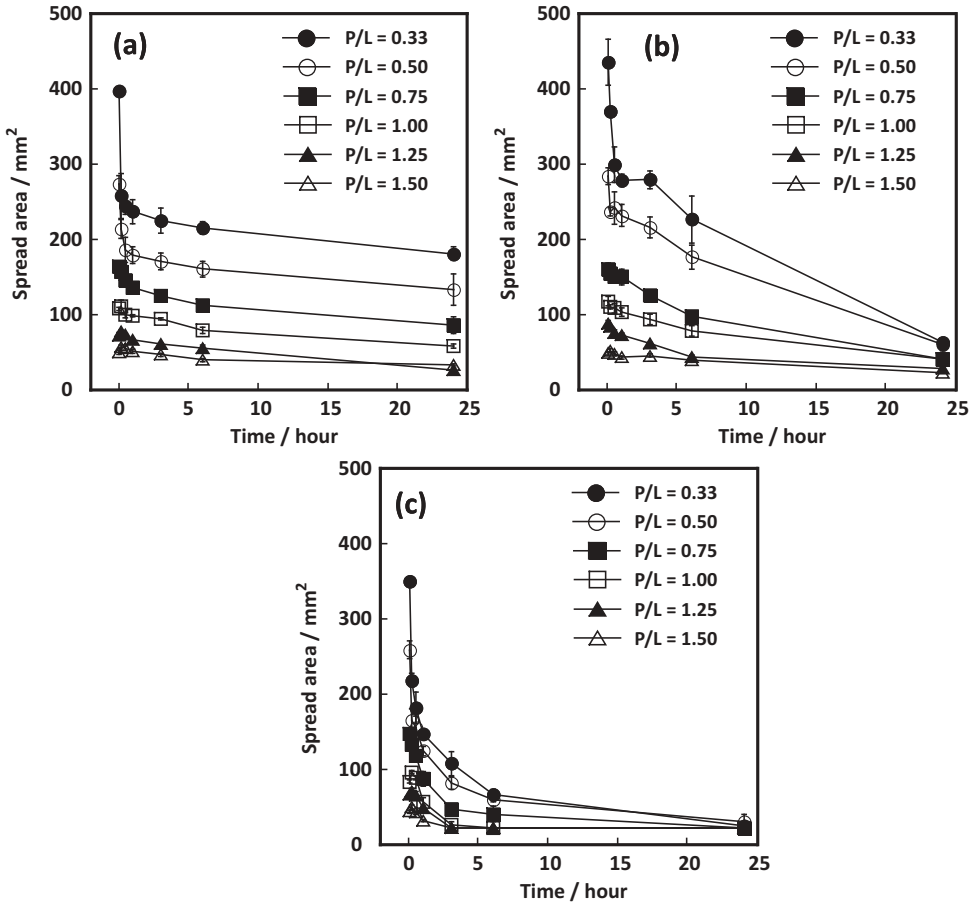

Figure 4. Time course of the pastes' viscosity: (a) G0.1-, (b) G1.0-, (c) G10-pastes.

3.4. 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 Figure 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 Figure 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.

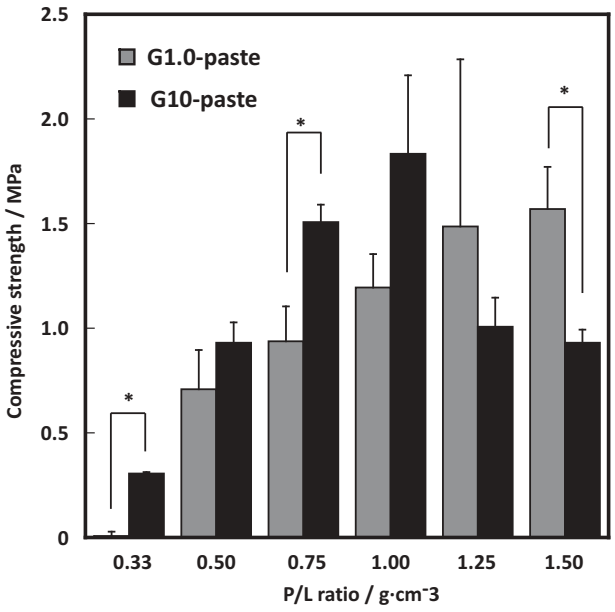

Figure 5. Compressive strength of the pastes other than the G0.1-pastes according to JIS.

3.5. Cytocompatibility test

3.5.1. Influences of GPTMS concentrations

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. Figure 7(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, whose safety is already confirmed by both animal tests and practical medical treatment of humans. G10-paste strongly inhibited

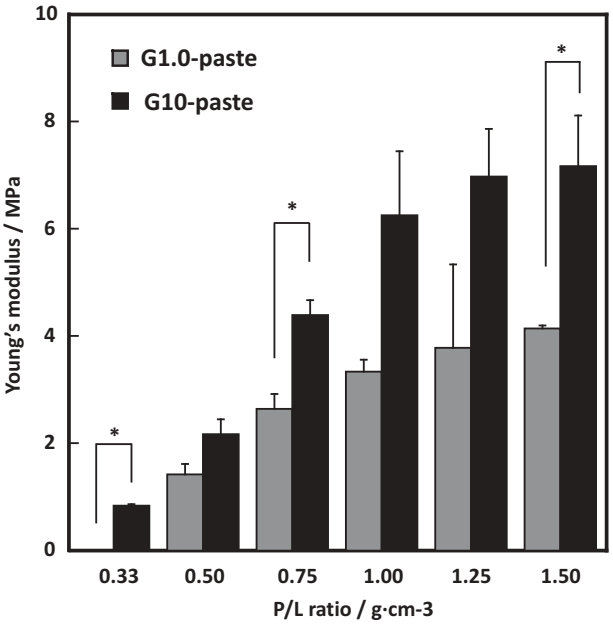

Figure 6. Young's modulus of the pastes calculated from the stress-strain curve of the compressive strength test, except for the G0.1-pastes.

cell proliferation in the first 2 days, and the results for 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.

3.5.2 Influence of P/L ratios

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 and hardening behavior at all the P/L ratios. As shown in Figure 8(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. Figure 8(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 G1.0-pastes at day 3. No Si ions were detected at day 7.

3.6. Preliminary confirmation of bone tissue reactions

The pig showed no systemic or local symptom during the test period. After sacrifice, naked-eye observation detected no paste implanted in the sites, and all the sites seemed to be regenerated completely by new bone formation. Figure 9 shows a μ-CT image of bone after harvest, and no obvious signs of the paste implanted site are to be found. The implanted sites were therefore estimated from photos taken at the time of implant surgery and from the faint contrast in the CT images indicated by the dotted-line circle. The paste implanted sites were completely substituted with newly formed bone as observed by μ-CT.

4. 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

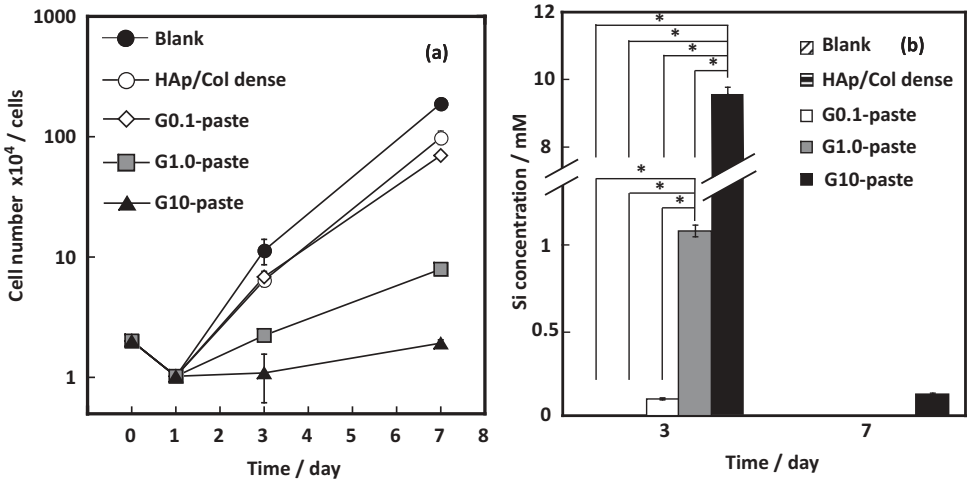

Figure 7. (a) Growth curve and (b) Si concentration of culture media cultured using P/L = 1.00 pastes.

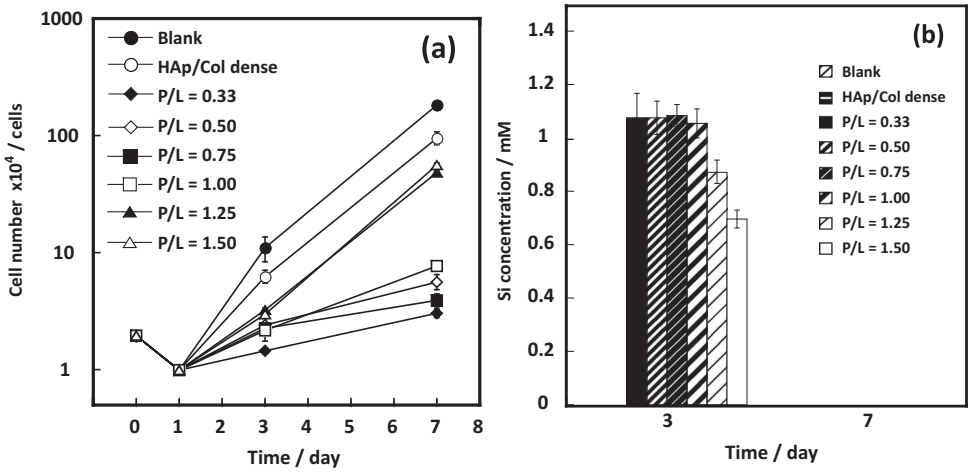

Figure 8. (a) Growth curve and (b) Si concentration of culture media cultured using G1.0-pastes.

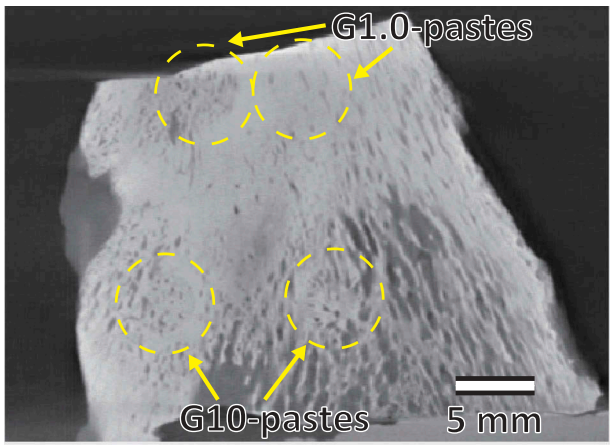

Figure 9. μ-CT image of G1.0- and G10-pastes implanted into a pig's tibia.

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 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 [28]. The relationship between the liquid volume of GPTMS for 1 g of HAp/Col powder and the spread area, shown in Figure 10, 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.

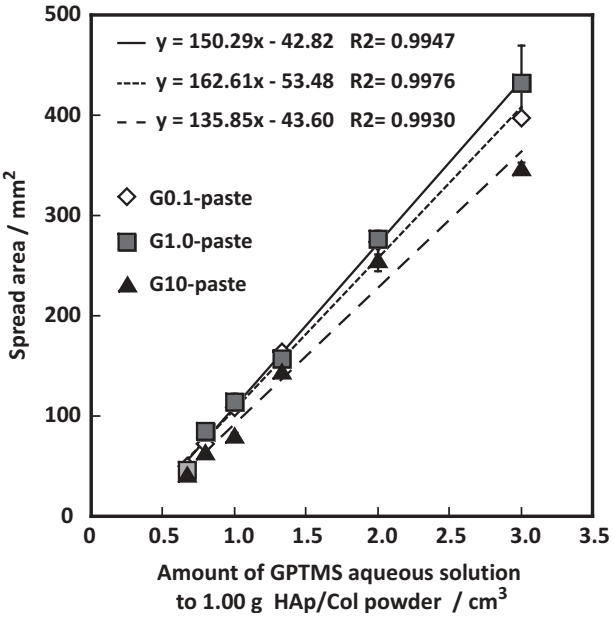

Figure 10. Correlation between the spread areas of the viscosity test and amount of the liquid phase of the pastes.

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 [29].

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 [16].
(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 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 [30,31], 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.

Since the timing of immersion of the test samples in the solution in the cytotoxicity test falls into the transition period from Step 1 to Step 2, the Si ion concentrations in the culture medium at day 3 are used to interpret the amounts of the GPTMS leached from the paste during the setting reaction, i.e. released from the paste in less than 24 h. 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 cytotoxicity test, the cytostatic activity was found from day 1 to day 3, the first medium change. Hence, the Si ion concentration, i.e. the eluted GPTMS concentration, was negatively related to cell proliferation. The GPTMS leached from the paste 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 sufficiently lower Si ion concentration, GPTMS amount, showed a few reduction effects on cell growth, as seen in cells cultured with the G10-paste at day 7. A conventional cell culture uses a very small amount of medium; a large amount of body fluid flows in the living tissues, however, and the effects of the eluted GPTMS may be limited. This assumption was confirmed to be correct in the preliminary *in vivo* test in the present study, as no obvious symptoms were observed in the test.

Naked-eye observation of the pastes and surrounding bone just after operation and surgery the

extraction illustrated that the pastes at the bone surface were completely substituted by bone. μ-CT observation also revealed no differences between the host bone and the paste. Theoretically, the X-ray absorption coefficients of the HAp/Col-GPTMS pastes were smaller those that of bone due to the greater amount of water contained. Thus, the pastes were completely resorbed and replaced with newly formed bone.

5. Conclusions

The pastes prepared from the HAp/Col powder and the GPTMS solution as described in this paper demonstrated sufficient injectable fluidity and anti-washout properties for practical use in medical and dental fields. The hardened pastes showed a viscoelastic nature. 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 to the porcine tibia. The animal test revealed that the pastes were bioresorbable and completely substituted by newly formed bone. Hence, HAp/Col-GPTMS pastes are good candidates for use as a novel bioresorbable bone void filler.

Disclosure statement

No potential conflict of interest was reported by the authors.

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