

Preparation of poly(3-hydroxybutyrate-co-4-hydroxybutyrate)-based composites releasing soluble silica for bone regeneration

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Silica/poly(3-hydroxybutyrate-co-4-hydroxybutyrate) composites were prepared for achievement of new medical devices having bioactivity, biodegradability and excellent mechanical-elongation property for the use in bone regeneration. The composites were prepared using three different types of silicon alcoxides, Tetraethylorthosilicate (TEOS), Diethoxydimethylsilane (DEODMS) and 3-aminopropyltriethoxysilane (APTES), as a silica source. The composites prepared with TEOS possessed both abilities of soluble silica releasing and hydroxyapatite forming in simulated body fluid, while the composites prepared with the other sources didn't show them. The chemical structure and tensile properties of the composites prepared with TEOS were evaluated. Fourier transform infrared reflection and ¹³C cross polarization magic angle spinning nuclear magnetic resonance spectroscopic analyses demonstrated that the crystallinity of polymer phase in the composites was reduced by adding the silica. The composites containing <25 wt % of silica exhibited a large failure strain (~700%).

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1. Introduction

Polyhydroxyalkanoates (PHA) are natural thermoplastics and generally biodegradable with excellent biocompatibility, which makes them attractive as biomaterials.¹⁾⁻⁵⁾ PHA are produced by microorganisms under unbalanced growth conditions. Several PHA have been developed so far, such as poly 3-hydroxybutyrate (PHB), copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), poly 4-hydroxybutyrate (P4HB), copolymers of 3-gydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) and poly 3-hydroxyoctanoate (PHO).^{1),6)} The main advantages of PHA over other types of biodegradable plastic are that they are able to be degraded regardless of the environmental conditions, e.g. they are rapidly degraded under aerobic and anaerobic conditions, and their in vivo breakdowns are considerably less acidic and less inflammatory than many currently used synthetic absorbable polymers such as poly(lactic acid). The degraded product is a normal component of blood or tissue.^{7),8)} PHB is one of the most popular PHA and has been investigated for the use in bone regeneration.⁹⁾ It is, however, brittle and has some other weak mechanical properties. The copolymer consisting of PHB and P4HB, i.e. P(3HB-co-4HB) [Fig. 1(a)], has been developed and investigated in its chemical structure and mechanical properties.^{9),10)} The degree of crystallinity of P(3HB-co-4HB) decreased with increase in the amount of the 4HB content, while other copolymer of PHA, such as PHBV, form isomorphic crystals in which some of the minor comonomer components co-crystallize with the major comonomer components.¹¹⁾ P(3HB-co-4HB) has a statistically random distribution of PHB and 4HB units. P(3HB-co-4HB) with a wide range of compositions from 0 to 100

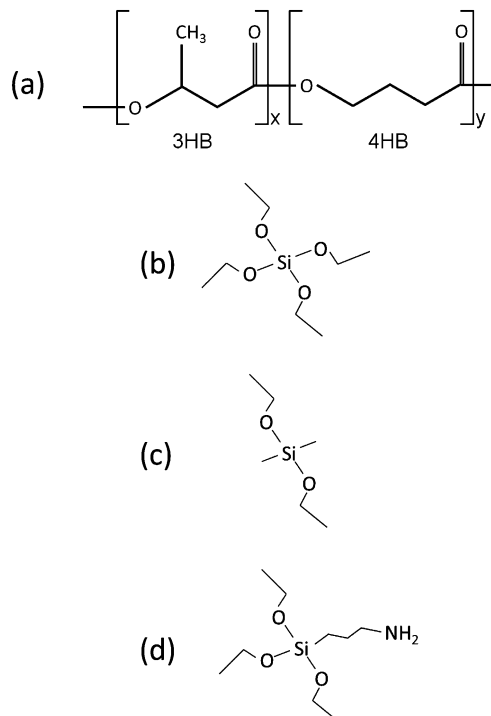


Fig. 1. Chemical structures of (a) P(3HB-co-4HB), (b) TEOS, (c) DEODMS and (d) APTES.

mol % 4HB have been synthesized and their biodegradability was reported to depend on the 4HB content in the copolymer. The mechanically tensile strength increased with increase in the 4HB content. These mean that the degradability and tensile property

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of the copolymer are able to be controlled by changing the 4HB content. The copolymer exhibits an excellent elongation, e.g. the elongation to failure was reported to be 1320% for the copolymer with 82 mol % 4HB.¹⁰⁾ The copolymer is expected to be useful to develop medical devices including implantable rods, drug delivery system, bone plates, surgical mesh, tissue engineering scaffolds and etc.^{6),8),12)} P(3HB-co-4HB), however, has a limitation in its application to the scaffolds for bone regeneration, because it has a low bioactivity.

Incorporating bioactive glasses into bioinert polymers is one of the useful methods to develop medical devices with bioactivity, elasticity, toughness, and so on.¹³⁾ Some inorganic elements show the enhancement effects on particularly bone tissue regeneration. Recently, ionic dissolution products from the bioactive glasses, such as silicon, calcium, phosphorous, magnesium, strontium, boron, vanadium and cobalt, have been reported to stimulate osteogenic cell functions, such as proliferation, differentiation and mineralization.¹⁴⁾⁻²²⁾ Especially the up-regulations of osteogenic cell functions by silicon have been widely reported. The aim of the present work was to synthesize silica/ P(3HB-co-4HB) composites with bioactivity and the ability of soluble silica releasing by a sol-gel method. In case of composites consisting of rigid filler and polymer matrix, their tensile properties depend on the distribution state of the filler in the matrix and the adhesion strength between the filler surface and the matrix.^{23),24)} Therefore, three different types of silicon alcoxides, Tetraethylorthosilicate (TEOS), Diethoxydimethylsilane (DEODMS) and 3-aminopropyltriethoxysilane (APTES), were used to synthesize the composites. The chemical structure of each alcoxide is shown in Fig. 1. The distribution state of silica derived from each alcoxide in the polymer matrix was observed and the abilities of hydroxyapatite (HA) forming in simulated body fluid (SBF)²⁵⁾ and soluble silica releasing in Tris buffer solution were evaluated. The composites with various contents of silica derived from TEOS were also prepared and their chemical structures and mechanically tensile properties were characterized.

2. Materials and methods

2.1 Sample preparation

Silica source, TEOS (Sigma-Aldrich, USA), DEODMS (Shin-Etsu Silicone, Japan) or APTES (Sigma-Aldrich, USA), was mixed with ethanol, distilled water (DW), 1M HCl solution at room temperature (RT) for 1 h. The chemical components of the solution were shown in **Table 1**. Polymer solution (5 wt %) was prepared using P(3HB-co-4HB) (4HB content; 18 mol %, G5, Japan) and chloroform. The silica source solution was added to the P(3HB-co-4HB) solution, and then they were mixed for 0.5 h at RT. The resultant solution was casted in a Teflon dish and dried at RT, resulting to the formation of a silica/P(3HB-co-4HB) composite film. Silica content in the composites was set to be 25 wt % for all samples. The composite films consisting of TEOS, DEODMS or APTES were denoted by 25T, 25D or 25A, respectively.

Table 1. Chemical component of silica source solutions for preparing P(3HB-co-4HB)-based composites

Silica source	Silica source:Ethanol:DW:HCl (mol ratio)	Silica content (wt %)	Resultant sample code
TEOS	1:4:2:0.01	25	25T
DEODMS	1:4:1:0.01	25	25D
APTES	1:4:1.5:0.01	25	25A

TEOS-series composites with various contents of silica were prepared with the aforementioned method. TEOS solution was prepared by mixing TEOS, ethanol, DW and 1M HCl solution with the molar ratio of 1:4:2:0.01. Resultant films containing 5–40 wt % of silica were termed 5–40 T, respectively.

2.2 Materials characterization

The structural analyses of composite films were performed using attenuated total reflectance Fourier transform infrared reflection spectroscopy (ATR-FTIR, FT/IR-4100, JASCO, Japan) and ¹³C cross polarization magic angle spinning nuclear magnetic resonance (¹³C CP/MAS-NMR, UNITY plus, Varian, USA). ¹³C CP/MAS-NMR spectra were taken with 7.2- μ s and 7.5-s recycle delays. Surface and cross-sectional morphology of the films was observed with scanning electron microscope (SEM, JSM-6301F, JEOL, Japan) after coating the samples with amorphous osmium layer using a vapor deposition method (Neoc, Meiwafoysis Co. Ltd., Japan). The films for the cross-sectional observation were cut with a scissors immediately after soaking in liquid nitrogen for a few minutes. Silica particle size formed in the films means the average value of 20 particles estimated from the SEM images.

The molecular weights of P(3HB-co-4HB) in the composites were measured by gel permeation chromatography (GPC, Shimadzu, Japan) equipped with two columns (K-806L, Shodex, Japan). The composites were dissolved in chloroform at the rate of 5 μ g of P(3HB-co-4HB) per 5 mL of chloroform and the solutions were filtered. The measurement was carried out at 40°C with 1.0 mL/min of the flow rate. Polystyrene standards (SM-105, Shodex, Japan) were used to establish the calibration curve.

The amount of silicon released from the films was evaluated by immersing them with the dimensions of 20 \times 20 \times 0.15 mm in 10 mL of Tris buffer solution (pH 7.4) and incubated at 37°C for \sim 7 days. The Si concentration of the buffer solution was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES, ICPS-500, Shimadzu, Japan). A calibration curve was established using Si⁴⁺ ion-containing aqueous solution with concentrations of 1, 25, and 50 ppm. Results mean the average value of 3 samples.

2.3 SBF immersion test

The film was immersed in 30 mL of SBF at 37°C for 3 days. The SBF solution consisting of 2.5 mM Ca²⁺, 142.0 mM Na⁺, 1.5 mM Mg²⁺, 5.0 mM K⁺, 148.3 mM Cl⁻, 4.2 mM HCO₃⁻, 1.0 mM HPO₄²⁻, 0.5 mM SO₄²⁻, 33.3 mM (CH₂OH)₃CNH₂ and 30.0 mM HCl was prepared using reagents of NaCl, NaHCO₃, KCl, K₂PO₄·3H₂O, MgCl₂·6H₂O, HCl, CaCl₂, NaSO₄ and NH₂C(CH₂OH)₃. After immersion in SBF solution, the film was washed with DW, and then dried at RT. The sample surfaces after the immersion were observed by SEM after coating with osmium and characterized by X-ray diffractometry (XRD, RAD-B, Rigaku, Japan) (CuK α , 40 kV, 20 mA).

2.4 Mechanical tests of TEOS-series composites

Tensile tests for the TEOS-series composite films were performed using an Autograph (AGS-G, Shimadzu, Japan) with a load cell (50 N). The dumbbell-shaped film having a rectangle with the dimension of 10 \times 5 \times 0.15 mm in its center was used for the test. Results mean the average values of 3 samples.

3. Results

3.1 Material characterization of 25T, 25D and 25A
Figure 2 shows ATR-FTIR spectra of 25T, 25D, 25A and

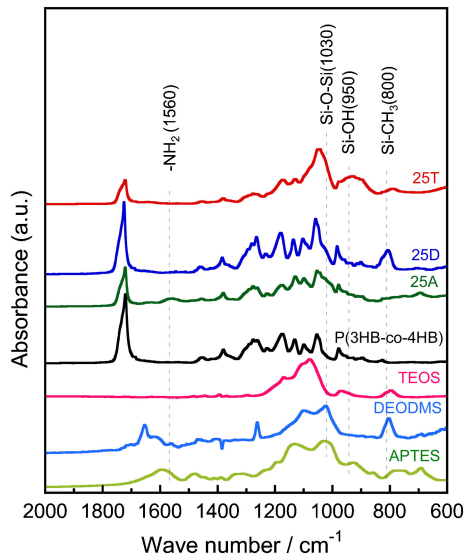


Fig. 2. ATR-FTIR spectra of composite samples and pure P(3HB-co-4HB). FTIR spectra (with KBr method) of silica sources are shown as references.

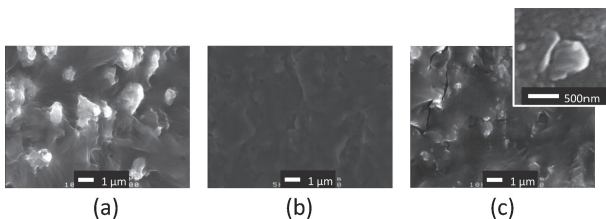


Fig. 3. Cross-sectional SEM images of (a) 25T, (b) 25D and (c) 25A. The inset of (c) shows the magnified particle in 25A.

Table 2. Weight-average molecular weight (Mw), number-average molecular weight (Mn), and polydispersity (Mw/Mn) of P(3HB-co-4HB) in composites

Sample code	Mw/kDa	Mn/kDa	Mw/Mn
P(3HB-co-4HB)	1039	399	2.60
25T	901	357	2.52
25D	924	329	2.80
25A	561	302	1.86

P(3HB-co-4HB). FTIR spectra (with KBr method) of silica sources were also shown as references. Silica network was formed in all the composites. Characteristic peaks of each silica source, $-\text{CH}_3$ for DEODMS and $-\text{NH}_2$ for APTES, were found in the spectrum of 25D and 25A, respectively. The peak corresponding to Si-OH was found to be higher on the spectrum of 25T than the others, when the peak intensity of C=O (in polymer structure) was used as a reference. SEM observation results (Fig. 3) demonstrated that silica particles were formed in 25T and 25A, while no particles were observed in 25D. The particle sizes of 25T and 25A were appropriately 1.2 and 0.6 μm , respectively. Table 2 shows the molecular weight and polydispersity of P(3HB-co-4HB) contained in each composite. Although the Mw values of 25T and 25D were almost the same (901 kDa for 25T and 924 kDa for 25D), the value of 25A (561 kDa) was lower than those of the other composites and appropriately half of that of P(3HB-co-4HB) (1039 kDa). The poly-

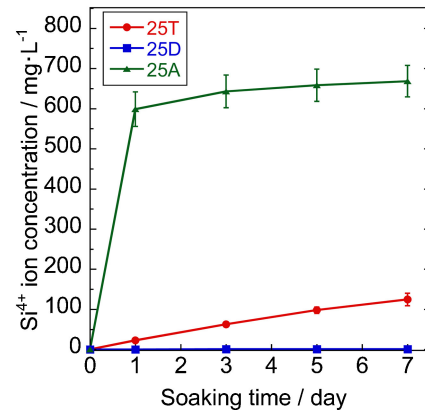


Fig. 4. Si^{4+} ion concentration dissolved from the samples in Tris buffer solution after soaking 1–7 days.

dispersity (Mw/Mn) of 25A was lower than those of the others. Figure 4 shows the Si ion concentration of Tris buffer solution after soaking the composite films for 1–7 days. The concentration value of 25A rapidly increased in 1 day after soaking, while the value of 25T linearly increased with the soaking time. No ion was released from 25D.

3.2 Hydroxyapatite formation in SBF

Figures 5(a)–5(f) show the surface morphology of the composite films after the immersion in SBF for 1 day or 3 days. Deposits with leaf-like shape were found on 25T surface and their amount increased with the soaking time. Pits with the size of several μm were formed on the whole surface of 25A, while no deposits were observed. The surface of 25D showed no obvious change in morphology compared with that before soaking. The deposits were believed to be HA from the XRD patterns of 25T [Fig. 5(g)]. The peak corresponding to HA should be present around 26° , however it was not observed on the pattern of the sample after soaking as the broad peaks corresponding to the polymer were higher.

3.3 Chemical structure and mechanical strength of TEOS-series composites

ATR-FTIR and ^{13}C CP/MAS-NMR spectra of the TEOS-series composites with the silica content range of 5–40 wt% (Figs. 6 and 7) demonstrated that chemical bonding was formed between silica and P(3HB-co-4HB). In the results of ATR-FTIR, the intensity of shoulder peak corresponding to C=O (amorphous)²⁶⁾ at 1740 cm^{-1} increased with the increase in the silica content. A shoulder peak was present at 1705 cm^{-1} in the spectra of the composites and its intensity increased with the increase in content, while the peak was not observed in that of P(3HB-co-4HB). The intensity of peaks corresponding to Si-O-Si and Si-OH increased with the increase in silica content. Figure 7 shows the ^{13}C CP/MAS-NMR spectra; (a) and (b) show the peaks corresponding to $-\text{C}=\text{O}$ and $-\text{CH}_3$, respectively. Solid line indicates the peak top position on the 0T [P(3HB-co-4HB)] spectrum. Dashed line indicates the top position of shifted peak. The $-\text{C}=\text{O}$ and $-\text{CH}_3$ peak shifted to lower and higher magnetic field, respectively, in the spectra of composites. Figure 8 shows the molecular weight and polydispersity measured by GPC. Mw decreased with the increase in the silica content, while the polydispersity (Mw/Mn) maintained almost the same value.

Figure 9 shows typical stress-strain curves of TEOS-series composites. The maximum stress (F_{max}) and elongation to fail-

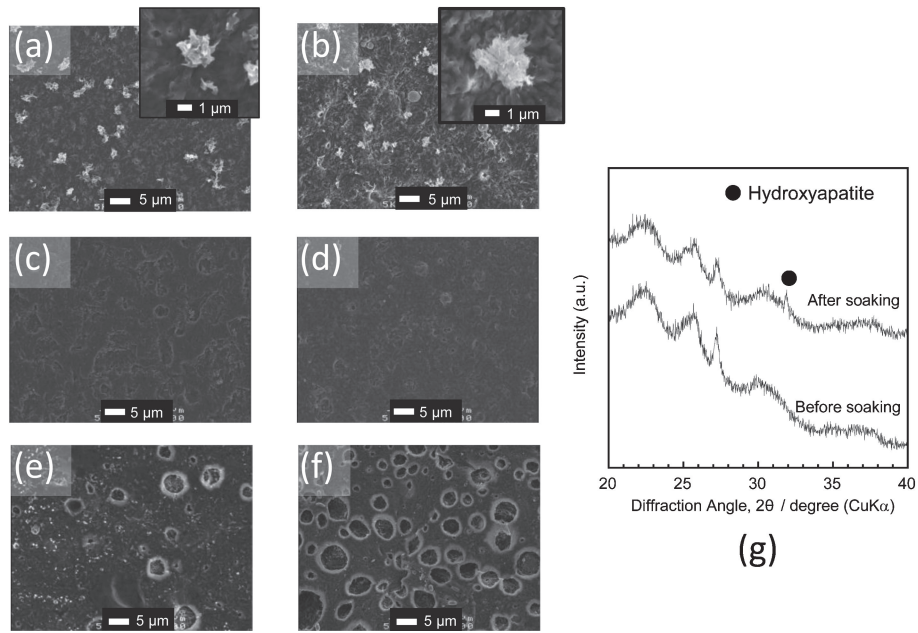


Fig. 5. SEM images of (a, b) 25T, (c, d) 25D and (e, f) 25A after (a, c, e) 1-d or (b, d, f) 3-d of soaking in SBF. The insets of (a, b) show the magnified deposits on 25T. (g) XRD patterns of 25T before and after 1-d soaking.

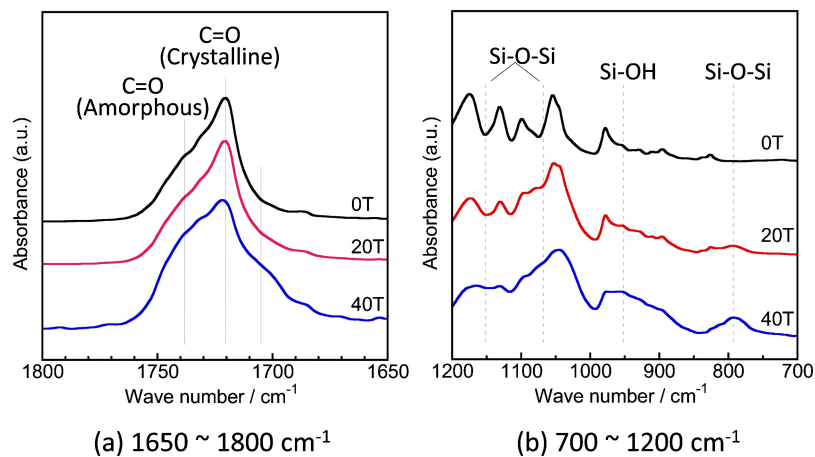


Fig. 6. ATR-FTIR spectra of the TEOS-series composites with various silica contents.

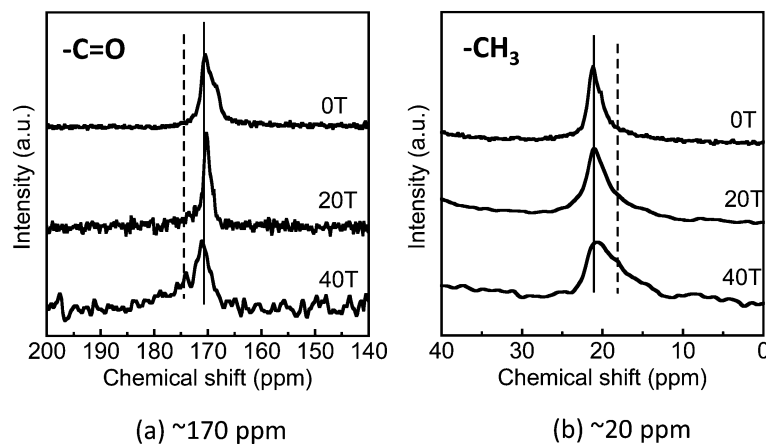


Fig. 7. ¹³C CP/MAS-NMR spectra of the TEOS-series composites with various silica contents. Solid line indicates the peak top position on the 0T spectrum. Dashed line indicates the position of shifted peak top.

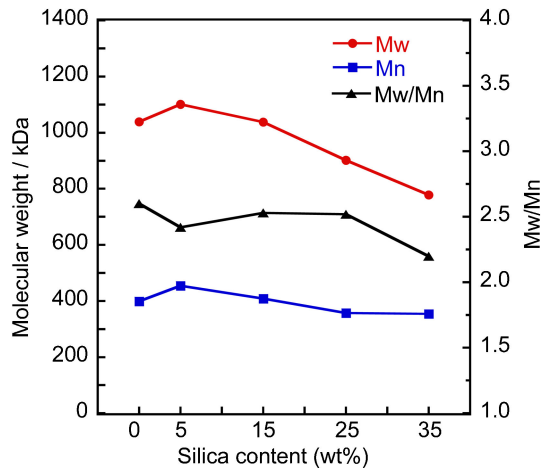


Fig. 8. Molecular weight and polydispersity of P(3HB-co-4HB) in TEOS-series composites.

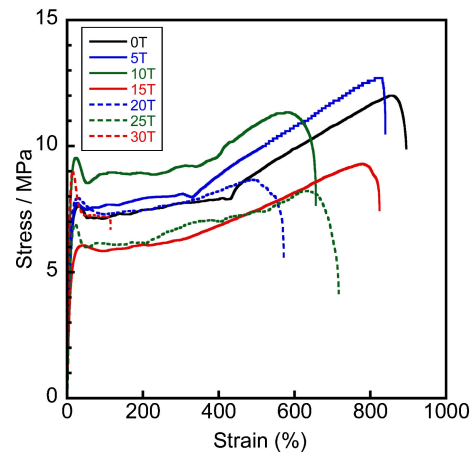


Fig. 9. Stress-strain curves of the TEOS-series composites with various silica contents.

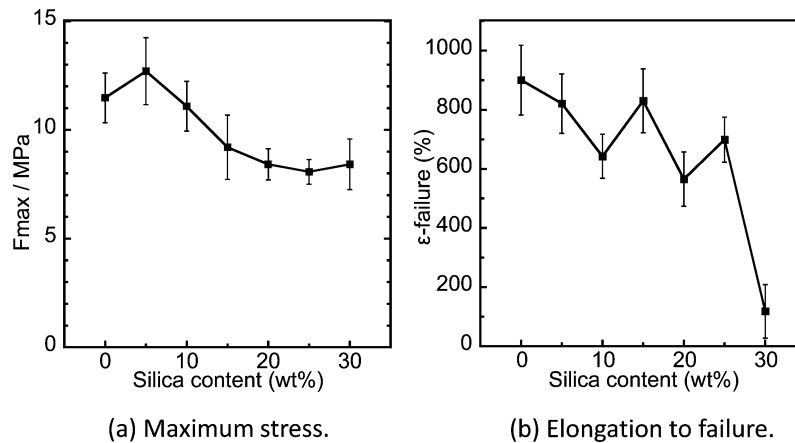


Fig. 10. Tensile properties of TEOS-series composites as a function of silica content.

ure (ϵ -failure) as a function of the silica content in the composites were plotted in Fig. 10. The F_{\max} was calculated from the stress value of the peak top which appears after the plastic deformation of the samples. The ϵ -failure was calculated from the strain value at which the extended line of each stress-strain curve reaches the x -axis. The F_{\max} value decreased with increasing the content. The ϵ -failure value drastically decreased in the range from 25 to 30 wt % in the content.

4. Discussion

Silica/P(3HB-co-4HB) composites were prepared using three different types of silicon alcoxides and their abilities of soluble silica releasing and HA forming were evaluated. SEM observation (Fig. 3) revealed that no particles were formed in 25D. The silica derived from DEODMS was linear and homogeneously dispersed in the polymer matrix because its hydrophobicity. The 25D released no Si^{4+} ion in Tris buffer solution. On the other hand, the silica derived from TEOS and APTES formed particles in the polymer matrix and released the ion in the solution. No obvious peaks suggesting any chemical interaction between each silica phase and the polymer matrix were observed in the ATR-FTIR spectra of the composites, except the peak corresponding to C=O (amorphous). The Si^{4+} ion release was thought to depend only on the solubility of each silica phase. The silica particle derived from APTES was smaller, immediately dissolved and finished releasing the ion in 1 day after soaking. Pits were formed

on the 25A surface after soaking in SBF. This was because the silica derived from APTES rapidly hydrolyzed and its hydrolysis products increased the surrounding pH, which accelerated the polymer degradation.²⁷⁾ The molecular weight of P(3HB-co-4HB) in 25A was lower than those of the other samples. The pH increase due to the added APTES induced the polymer degradation during the composite preparation. On the other hand, HA formed on the 25T surface in SBF and the molecular weight of 25T was almost the same as that of P(3HB-co-4HB). The silanol groups on the silica particles in 25T would induce the HA formation in SBF.^{28),29)} Thus, TEOS was regarded to be a good candidate for the silica source to prepare the composites.

The composites with various contents of silica derived from TEOS were prepared and their chemical structures and tensile properties were characterized. ATR-FTIR results demonstrated that the crystallinity of PHB phase in the composites was reduced by adding the silica. The CH_3 and C=O groups form C-H...O=C hydrogen bonding in the crystalline of PHB.²⁶⁾ The intensity of peak at 1705 cm^{-1} increased with the increase in the silica content. This peak was expected to correspond to the chemical reaction between the silica and the polymer chain, i.e. Si-OH...C=O hydrogen bonding. The ^{13}C CP/MAS-NMR spectra followed this speculation. The peak corresponding to -C=O shifted to lower magnetic field in the spectra of composites. Thus, it was supposed that no strong bonding formed between the silica and matrix.

The results of tensile test revealed that the maximum stress slightly decreased with the increase in the silica content. This might be due to void formation in the composites.²⁴⁾ On the other hand, the failure strain drastically decreased in the range from 25 to 30 wt % in the content. This phenomenon was attributed to the reduction in the amount of ductile polymer present. The volume % of the silica was estimated to be 17 for 25T and 20 for 30T (the density of the used silica and P(3HB-co-4HB) was 2.1 and 1.25 g/cm³, respectively). In our fundamental study, the failure strain of the composite consisting of P(3HB-co-4HB) and calcium carbonate particle drastically decreased with increasing the particle content from 17 to 24 vol %. Therefore we expected that the filler volume content in P(3HB-co-4HB) should be <20 vol % to achieve the composites with excellent elongation.

5. Conclusion

Silica/P(3HB-co-4HB) composites were prepared for achievement of new medical devices having bioactivity, biodegradability and excellent elongation property for the use in bone regeneration. Three different types of silicon alcoxides, Tetraethyl orthosilicate (TEOS), Diethoxydimethylsilane (DEODMS) and 3-aminopropyltriethoxysilane (APTES), were used as a silica source to prepare the composites by a sol-gel method. Only the composite prepared using TEOS showed soluble silica release in Tris buffer solution and hydroxyapatite formation in simulated body fluid. The chemical structure of the composites prepared using TEOS was evaluated ATR-FTIR and ¹³C CP/MAS-NMR. The crystallinity of PHB phase in the composites was reduced by adding the silica. The chemical reaction was expected to take place between the silica and the polymer chain, i.e. Si-OH...C=O hydrogen bonding. The composites containing <25 wt % (<20 vol %) of silica exhibited a large failure strain (~700%).

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