Preparation and In Vivo Study of Porous Titanium–Polyglycolide Composite

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Porous materials show low Young's moduli and excellent bonding to living bone. However, the strength of such materials is often insufficient in the initial stage of implantation. Thus, the objective of this study was to increase the strength of porous titanium by filling the pores with polyglycolide (PGA), a biodegradable plastic. PGA powder was prepared via the thermal decomposition of sodium chloroacetate at 433 K. The PGA was then introduced into the pores of porous Ti (porosity: 60%) using two methods: (i) centrifugal packing and heating and (ii) heat injection. In the latter method, almost all pores were filled by PGA; the filling fraction was measured to be 65–85% regardless of the injection temperature. When the pores in the porous Ti were filled with PGA, the compressive strength increased drastically from 40 to 100 MPa. The increased strength is comparable to that of cortical bone. In addition, the strength increased with increasing injection temperature. In an animal test, unfavourable autopsy findings, such as suppuration, bleeding, and hyperplasia of the connective tissue, could not be confirmed in rats and no bone was observed in the pores of the Ti–PGA composite. Decomposition of PGA lowered the surrounding pH, it was found to inhibit bone formation in the pores of the porous Ti. It is important to control the decomposition rate of PGA. [doi:10.2320/matertrans.MI201502]

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1. Introduction

Metallic materials are widely used as biomaterials for orthopaedic and dental applications because of their excellent mechanical properties and reliability. In orthopaedic surgery, however, the large mismatch between the Young's moduli of metallic implants and bone poses a serious problem. For example, the Young's moduli of pure Ti and cortical bone are 110 and 10–30 GPa, respectively. As a result of this mismatch, the bone around such metallic implants is insufficiently loaded, thus causing bone atrophy¹⁾. This phenomenon, called 'stress-shielding', has been studied by many researchers.

The mechanical properties of metallic materials can be controlled by both alloy design^{2–4)} and the construction of an appropriate structure^{5–8)}. A porous implant material with an adequate pore structure showing appropriate mechanical properties, a low Young's modulus, and bone ingrowth has long been sought as the ideal bone substitute⁹⁾. The mechanical properties of porous materials change from day to day; the bone ingrowth into the pores causes the Young's modulus to increase significantly after implantation. Therefore, it is necessary to implant porous devices with lower Young's moduli than that of living bone to mechanically fit the bone after complete healing. In this case, the strength also drastically decreases from the ingot state; thus, the strength must be insufficient in the initial stage of implantation.

Figure 1 shows a schematic of the present material design of a porous device, focusing on the compressive strength plotted against the volume fraction of ingrown bone, which is equivalent to the porosity of the base Ti matrix. In other

Fig. 1 Schematic illustration of change in compressive strength of Ti with porosity and the rule of mixtures applied to the porous Ti and bone.

words, this is a device design that takes into account the changes in the mechanical properties due to bone ingrowth. Several properties such as compressive, tensile and bending strengths are very important in actual devices for bone substitutes. In this study, however, the compressive strength was focused as a representative for the properties. The compressive strength of pure Ti (ASTM grade 2) is 275–410 MPa, and it decreases with increasing porosity, as shown by the solid line. For example, sintered Ti with 60% porosity actually exhibits a compressive strength of 40 MPa. Conversely, cortical bone exhibits a strength of 100–200 MPa. If the pores in porous Ti are filled by ingrown bone with a strength of 100 MPa, the implanted device is expected to exhibit a strength of 100 MPa simply by the rule of mixtures. As a result, the implant must be compatible with the surrounding bone after complete healing. As mentioned above, however, the initial strength must be improved to become equivalent to that of the bone. In the present design, of course, any Ti alloys e.g. Ti-6Al-4V can be employed instead of the pure Ti by adjusting the pore structure to obtain suitable mechanical

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properties.

Polyglycolide (PGA) is a polyester with a melting point of 493–503 K. The polymer is non-toxic, has good mechanical properties, and can be readily metabolised by the body. Therefore, this polymer is used extensively in medical applications such as drug delivery and resorbable implants¹⁰⁾ and has been studied in depth in the 1990s^{11–20)}. PGA can be produced via the following exothermic solid-state polycondensation reaction of sodium chloroacetate between 373 and 473 K:

Sodium chloride (NaCl) in the form of micrometre-sized cubes is a by-product of this reaction¹¹⁾ and can be easily removed by washing with water^{12,14)}.

The objective of the present study was to increase the strength by filling the pores of porous Ti with the biodegradable material, PGA. If the incorporated material is subsequently replaced by newly formed bone, the strength of the device would remain constant from just after implantation until complete healing. In addition, ingrown bone in animal tests was also investigated.

2. Experimental

2.1 Materials

A mixture of pure Ti powder (ASTM grade 2, gas atomised, average particle size < 45 μm) and ammonium hydrogen carbonate was shaped under a uniaxial compression of 100 MPa and used as a space holder. The green compact was sintered at 1673 K for 7.2 ks under argon atmosphere. The porosity was set to 60%, which is the lowest value that all pores are continuous. Under this condition, the Ti scaffold may be completely integrated with the bone by its ingrowth into the pores. In addition, it is easy to estimate the strength after complete healing due to the rule of mixtures. The cylindrical samples were cut from this compact by electro-discharge machining in deionised water. The obtained samples (Ø2 or 3 mm $\times$ 5 mm) were annealed at 1273 K for 3.6 ks under an argon atmosphere to remove the oxidised layer on the surfaces.

Sodium chloroacetate ($\text{ClCH}_2\text{COONa}$, 98% purity, Sigma Aldrich) was spread thinly over a Petri dish to avoid excessive heat build-up during fabrication. The Petri dish was placed in an oven, heated to 433 K in a vacuum to prevent the oxidation of the resulting polymer, and held for 72 ks (20 h). NaCl was removed from the product polymer by stirring it in distilled water for 5 h. The resultant PGA powder was rinsed with ethanol twice and vacuum-dried at 353 K.

2.2 Preparation of porous Ti–polyglycolide composites

In this study, PGA was introduced into the pores of porous Ti using two methods: (i) centrifugal packing and heating and (ii) heat injection. In the centrifugal packing and heating method, PGA powder was dispersed in distilled water to obtain a PGA slurry. The concentration was set to 0.03–0.10 g/mL. The cylindrical sample of porous Ti was soaked in the slurry in a spitz tube. A centrifugal force of 3750 G (G: gravitational acceleration, 1 G = 9.8 m/s²) was then applied to the tube using a conventional centrifugal machine. After drying, the samples were heat treated at 498 K for 300 s to melt the

PGA. In the heat injection method, the porous Ti and PGA powder were placed in a die made of stainless steel and heated to 483–513 K using high-frequency induction heating equipment. The heating rate was 0.167 K/s (10 K/min). The melted PGA that buried the porous Ti was compressed with a pressure of up to 2 MPa. After 300 s, the die and the sample were furnace-cooled. In both methods, extra PGA on the surfaces of composites was carefully removed with emery paper.

2.3 Characterisation

X-ray diffraction ($\text{CuK}\alpha$ radiation) analysis was performed using a Rigaku RINT 2500 diffractometer to identify the crystal structure of the fabricated polymer. The X-ray generator was operated at 40 kV and 300 mA. Data were collected over 2θ values ranging from 10° to 50° with a step size of 0.02°.

PGA powder (2–10 mg) was held in an aluminium crucible with its lid crimped in place. An empty aluminium pan was used as a reference. A temperature scan between 313 and 523 K was performed at a heating rate of 0.167 K/s (10 K/min) using a Shimadzu DSC-60 Plus to examine the melting behaviour of PGA.

A compression test was conducted at an initial strain rate of $1.67 \times 10^{-4} \text{ s}^{-1}$ for the porous Ti and the porous Ti–PGA composites using a Shimadzu Autograph AG-X Plus with a 5-kN load cell. The 0.2% proof stresses were determined from the stress–strain curves. Cylindrical samples (Ø3 mm $\times$ 5 mm) were used in this mechanical test.

The composite was immersed in 10 mL of distilled water, which was maintained at 310 K (37°C). After being soaked for different periods of up to 10 days, the changes in the weight of the composite and the pH of the solution were investigated.

Seven-week-old male Sprague Dawley rats (Charles River Laboratories Japan, Inc., Tokyo, Japan) and TiO_2 -coated porous Ti (Ø2 mm $\times$ 5 mm) were used in the present animal study. The details of the surface modification were reported in previous papers^{21,22)}. The samples were implanted in the tibial metaphyses of the rats. The rats were sacrificed after four weeks, and the implants were retrieved with the surrounding tissue. Following polymerisation, each implant block was sectioned and polished into 20- μm -thick slices. These sections were then stained with Villanueva–Goldner stain. Histological observation was performed by optical microscopy.

3. Results and Discussion

3.1 Characteristics of resultant polyglycolide powder

In this study, PGA was fabricated from sodium chloroacetate by thermal decomposition to obtain a PGA with a low molecular weight that shows rapid decomposition. The molecular weight of the PGA fabricated using this method is 2300 g/mol¹³⁾, which is reported to be much lower than that of PGA formed by other methods. The PGA fabricated by this method is reported to release 100% of a loaded drug within two days, whereas PGA with a higher molecular weight requires between five and 15 days to release the same amount of drug¹⁰⁾.

The resultant powder was determined to be PGA by comparison with a previously calculated X-ray diffraction pat-

Fig. 2 (a) X-ray diffraction pattern of the present PGA powder and (b) the X-ray diffraction pattern of PGA calculated in a previous study²³⁾.

Fig. 3 Temperature scan of fabricated PGA at a heating rate of 0.167 K/s (10 K/min).

tern²³⁾, as shown in Fig. 2. In addition, it was confirmed that the PGA retained its structure at temperatures of up to 513 K.

A temperature scan between 313 and 523 K was performed at a heating rate of 0.167 K/s (10 K/min) in a differential scanning calorimeter (Fig. 3). An endothermic peak related to melting of fabricated PGA was observed with onset, peak, and finish temperatures of 440, 479, and 488 K, respectively. A small shoulder is visible on the left hand side of the peak, suggesting that two types of reactions may have occurred. It is thought that the portion with low crystallinity melted first, followed by the portion with high crystallinity. The crystallinity of the fabricated PGA must be low in the initial stages and must improve during the heating process.

As a result, the heating temperature for the preparation of the porous Ti–PGA composites was determined to be 483–513 K.

3.2 Mechanical properties of composites

As stated in Section 2.2, the porous Ti–PGA composites were prepared using two methods: (i) centrifugal packing and heat treatment and (ii) heat injection. Cross-sectional observation, measurement of the filling fraction, and mechanical testing were conducted to optimise the preparation conditions for the composites. The filling fraction of PGA powder in the pores was calculated as

Fig. 4 Cross-sectional images of (a) porous Ti and porous Ti–PGA composites prepared by (b) centrifugal packing, (c) centrifugal packing & heat treatment, and (d) heat injection. The Ti, PGA, and cavity regions are labelled 'T', 'P', and 'C', respectively.

$$\text{Filling fraction (\%)} = \frac{w_{\text{comp}} - w_{\text{pTi}}}{d_{\text{PGA}} \left\{ V_{\text{pTi}} - \left(\frac{w_{\text{pTi}}}{d_{\text{Ti}}} \right) \right\}}, \quad (2)$$

where w_{comp} is the weight of the composite, w_{pTi} is the weight of the porous Ti, V_{pTi} is the nominal volume of the porous Ti, d_{PGA} is the density of the PGA, and d_{Ti} is the density of the Ti.

Figure 4 shows cross-sectional images of the porous Ti and porous Ti–PGA composites. The divided and the polished planes of composites were observed in the samples corresponding to (a, b) and (c, d), respectively. In the sintered porous Ti, all pores were continuous, as shown in Fig. 4(a). When centrifugal packing was used, the pores were densely packed with PGA powder (Fig. 4(b)). However, large cavities caused by solidification shrinkage were observed in the pores after heat treatment, as shown in Fig. 4(c). The PGA filling fraction was only 10–12% when centrifugal packing was used. Conversely, when the heat injection method was used, all pores on the surface were filled by PGA. Furthermore, the PGA penetrated to the centre, though few small cavities were still confirmed (Fig. 4(d)). The filling fraction was measured to be approximately 65–85% when heat injection was used. No changes in shape and size of the pores were observed after any treatment including the surface modification.

The 0.2% proof stresses were obtained from the stress–strain curves, as shown in Fig. 5. All stress–strain curves were typical and smooth, as with ductile metals, even in the composites. The porous Ti showed a proof stress of approximately 40 MPa. The compressive strength increased when the pores were filled with PGA, even when centrifugal packing was used. Furthermore, it reached more than 100 MPa in the composite prepared by heat injection. This strength is comparable to that of cortical bone. The Young's modulus for the specimens also increased by the same order of magnitude as the strength.

Porous devices having a sufficient strength may be prepared by using Ti alloys even without incorporate materials. In such devices, however, the Young's modulus will be excessively high after bone ingrowth compared to that of surrounding bone. Therefore the present design of device, that incor-

Fig. 5 Proof stress in porous Ti and porous Ti-PGA composites prepared using different methods.

Fig. 6 Proof stress of the composites as a function of filling fraction of PGA in pores of porous Ti.

porated degradable material is replaced by bone in the body, may be the only way to keep mechanical properties constant in the entire healing period.

The proof stresses in the composites are plotted against the filling fraction of PGA in Fig. 6. As mentioned above, the filling fraction of PGA achieved by the centrifugal packing and heating method was approximately 10–12%. However, a higher fraction can sometimes be obtained by heating samples buried in PGA powder, though the reproducibility was very low. In this case, melted PGA may be sucked into the pores from PGA surroundings through neighbouring pores by capillary action. Unfortunately, the filling fraction could not be arbitrarily controlled. Therefore, such results are enclosed in parentheses in Fig. 6. The proof stress increased with increasing filling fraction.

Conversely, a high PGA filling fraction of 65–85% was obtained with good reproducibility when using the heat injection method. Interestingly, the strength increased with increasing injection temperature and constant filling fraction. The increase may have been due to the progressing crystallisation of PGA during the cooling process after the injection.

The present device was designed to gradually decrease its strength in the body through the decomposition of incorporat-

Fig. 7 Changes in weight of composite and pH of solvent after soaking the composite in distilled water. The composite was prepared by the heat injection at 483 K.

ed PGA. Therefore, the decomposition rate is very important. In addition, the decomposition of PGA is known to result in decreasing pH. Thus, changes in the weight of the composite and the pH of the solvent were examined by immersing the sample in distilled water for 10 days. This immersion test was also carried out in buffer solutions. Because the tests in buffer solutions and distilled water showed similar results, distilled water was selected as the soaking media in the present study for the sake of simplicity and experimental efficiency.

The weight of the composite decreased rapidly to 80% in the first two days of immersion and then gradually decreased to approximately 70%, as shown in Fig. 7. The proof stress decreased to approximately 50 MPa after immersion for one day, and this value was maintained throughout the remainder of the 10 days. As mentioned above, the initial proof stress for the composites varied depending on the injection temperature. However, all composites prepared at different temperatures showed similar tendencies in the soaking test. The PGA was observed to decompose from the portion with low crystallinity, whereas the portion with high crystallinity remained. The decrease in the strength is thought to be sensitive to even small changes in the volume of the filling material as decomposition progresses. With the decomposition of PGA, the pH of media rapidly decreased to 3 after only one day of immersion. As with the tendency of the weight change, the pH also showed no remarkable change between two and 10 days of immersion. To achieve an effective design for proposed devices, it is important to suppress the initial PGA decomposition. For examples, a crystallinity control of degradable materials by heat treatments or incorporation of basic materials is an effective method for improving the degradation characteristics^{24–28}.

Recently, an additive manufacturing has been received considerable attentions in several fields, because it has a lot of flexibility in the structural design. For examples, the internal/external porosities of metallic scaffolds can be separately and arbitrarily controlled. It may be a powerful and effective technique to control the mechanical properties of the metallic scaffold itself and the composite, and the decomposition behaviour of incorporated materials of the composites.

Fig. 8 Light microscopy histological sections in (a) porous Ti and (b) porous Ti-PGA composite after four weeks of implantation stained with Villanueva–Goldner stain. Green, red, and black regions correspond to calcified bone, osteoid, and Ti, respectively.

3.3 Histological analysis

In the present *in vivo* test, the smaller cylindrical porous Ti sample ($\varnothing 2\text{ mm} \times 5\text{ mm}$) was used after TiO_2 coating by chemical–hydrothermal treatment^{21,22}. The porous Ti–PGA composites were prepared by heat injection at 483 K. Unfavourable autopsy findings, such as suppuration, bleeding, and hyperplasia of the connective tissue, could not be confirmed in the rats and the implanted parts.

The present histological analysis employed Villanueva–Goldner staining for newly formed bone around implanted porous Ti and porous Ti–PGA composites. This type of staining allows the clear observation of newly formed bone in relevant parts; it stains the osteoid red and the mineralised bone green, as shown in Fig. 8. In the porous, an ingrowth of mineralised bone was confirmed to occur from the cortical bone to the pores, as shown in Fig. 8(a). The osteoid was also penetrated where it came into contact with cancellous bone or bone marrow. In contrast, nothing was observed in the pores of the porous Ti–PGA composite, as shown in Fig. 8(b). In addition, it was confirmed that the soft tissue was covered to isolate the implant from the body. As is well known, the decomposition of PGA causes the pH of the surrounding region to decrease, which might prevent the invasion of cells into the pores of the porous Ti. The suppression of PGA decomposition must improve bone formation in pores.

To quantitatively analyse the histology, an image analysis was carried out using ImageJ, a public domain, Java-based image processing program developed at the National Institutes of Health^{29,30}. The areas of calcified bone or osteoid were extracted and measured under the following conditions. The calcified bone region was extracted by setting the hue, saturation, and brightness to 0–106, 25–255, and 71–255, respectively. Additionally, the osteoid region was extracted by setting the hue, saturation, and brightness to 181–255, 106–255, and 0–162, respectively. Under these settings, the pore occupancies by the tissues were obtained separately in cortical bone and medullary cavity regions, as shown in Fig. 9. For example, vigorous bone formation was quantitatively evaluated in the vicinity of cortical bone. The pore occupancy reached approximately 30%, whereas it was a few percent in the porous Ti–PGA composite. Although the low pH interfered with the bone formation in the pores, the modified surface of the Ti was confirmed to help bone growth. Thus, it is possible to quantitatively analyse the bone histology using this method.

In the present composites, the problem to solve is the rapid

Fig. 9 Pore occupancy by calcified bone or osteoid in the cortical bone and medullary cavity from optical micrographs measured by ImageJ.

decrease in pH. A decomposition rate of degradable plastics such as PLA and PGA can be suppressed by using high-molecular-weight ones and incorporation of basic materials^{10,24–28,31}. If calcium phosphates or calcium carbonates are employed as the basic materials, the bone ingrowth could be promoted by the calcium release. As a result, it might realise both the bone ingrowth and the ideal change in mechanical properties.

4. Summary

- (1) PGA with a low molecular weight was fabricated from sodium chloroacetate by thermal decomposition. The onset and finish melting temperatures in the heating process were 440 and 488 K, respectively.
- (2) Porous Ti–PGA composites were prepared using a heat injection method with good reproducibility. The PGA penetrated to the centre of porous Ti showing 60% porosity. The filling fraction of the PGA was approximately 65–85%. The compressive strength of the composite exceeded 100 MPa.
- (3) The area fractions of calcified bone and osteoid were quantitatively analysed using ImageJ on the light microscopy histological sections in the porous Ti and porous Ti–PGA composites. Vigorous bone formation was confirmed in the vicinity of cortical bone. However, complexed PGA inhibited bone formation in the pores of the porous Ti.

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REFERENCES

- (1) N. Sumitomo, K. Noritake, T. Hattori, K. Morikawa, S. Niwa, K. Sato and M. Niinomi: *J. Mater. Sci. Mater. Med.* **19** (2008) 1581–1586.
- (2) M. Niinomi, M. Nakai and J. Hieda: *Acta Biomater.* **8** (2012) 3888–3903.
- (3) T. Ahmed, M. Long, J. Silvestri, C. Ruiz and H.J. Rack: in *Titanium'95: Science and Technology*, P.A. Blenkinsop, W.J. Evans and H.M. Flower, eds., The Institute for Materials, Birmingham, UK (1996). pp. 1760–1767.
- (4) D. Kuroda, M. Niinomi, M. Morinaga, Y. Kato and T. Yashiro: *Mater.*

- Sci. Eng. A **243** (1998) 244–249.
- 5) N. Nomura, T. Kohama, I.H. Oh, S. Hanada, A. Chiba, M. Kanehira and K. Sasaki: *Mater. Sci. Eng. C* **25** (2005) 330–335.
- 6) C.E. Wen, M. Mabuchi, Y. Yamada, K. Shimojima, Y. Chino and T. Asahina: *Scr. Mater.* **45** (2001) 1147–1153.
- 7) I.K. Oh, N. Nomura and S. Hanada: *Mater. Trans.* **43** (2002) 443–446.
- 8) H. Li, S.M. Oppenheimer, S.I. Stupp, D.C. Dunand and L.C. Brinson: *Mater. Trans.* **45** (2004) 1124–1131.
- 9) M. Takemoto, S. Fujibayashi, M. Neo, J. Suzuki, T. Kokubo and T. Nakamura: *Biomaterials* **30** (2005) 6014–6023.
- 10) J. Braunecker, M. Baba, G.E. Milroy and R.E. Cameron: *Int. J. Pharm.* **282** (2004) 19–34.
- 11) O. Herzberg, R. Gehrke and M. Epple: *Polymer* **40** (1999) 507–511.
- 12) M. Epple and O. Herzberg: *J. Mater. Chem.* **7** (1997) 1037–1042.
- 13) K. Schwarz and M. Epple: *Macromol. Chem. Phys.* **200** (1999) 2221–2229.
- 14) M. Epple and H. Kirschnick: *Chem. Ber.* **129** (1996) 1123–1129.
- 15) M. Epple and O. Herzberg: *J. Biomed. Mater. Res. (Appl. Biomater.)* **43** (1998) 83–88.
- 16) M. Epple and L. Tröger: *J. Chem. Soc., Dalton Trans.* (1996) 11–16.
- 17) K. Schwarz and M. Epple: *J. Phys. Chem. B* **106** (2002) 10764–10770.
- 18) L. Elizabé, B.M. Kariuki, K.D.M. Harris, M. Tremayne, M. Epple and J.M. Thomas: *J. Phys. Chem. B* **101** (1997) 8827–8831.
- 19) A.A. Ignatius and L.E. Claes: *Biomaterials* **17** (1996) 831–839.
- 20) K.D.M. Harris and E.Y. Cheung: *Chem. Soc. Rev.* **33** (2004) 526–538.
- 21) M. Ueda, R. Matsunaga, M. Ikeda and M. Ogawa: *Mater. Trans.* **49** (2008) 1706–1709.
- 22) M. Ueda, M. Ikeda and M. Ogawa: *Mater. Sci. Eng. C* **29** (2009) 994–1000.
- 23) Y. Chatani, K. Suehiro, Y. Okita, H. Tadokoro and K. Chujo: *Makromol. Chem.* **113** (1968) 215–229.
- 24) J. Liuyun, X. Chengdong, J. Lixin, C. Dongliang and L. Qing: *Mater. Res. Bull.* **48** (2013) 1233–1238.
- 25) C. Migliaresi, A.D. Lollis, L. Fambri and D. Cohn: *Clin. Mater.* **8** (1991) 111–118.
- 26) N. Fukuda, H. Tsuji and Y. Ohnishi: *Polym. Degrad. Stabil.* **78** (2002) 119–127.
- 27) M. Jayabalan, K.T. Shalumon, M.K. Mitha, K. Ganesan and M. Epple: *Acta Biomater.* **6** (2010) 763–775.
- 28) Y.S. Liu, Q.L. Huang, A. Kienzle, W.E.G. Müller and Q.L. Feng: *Mater. Sci. Eng. C* **38** (2014) 227–234.
- 29) W.S. Rasband, ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, (1997–2012).
- 30) C.A. Schneider, W.S. Rasband and K.W. Eliceiri: *Nat. Methods* **9** (2012) 671–675.
- 31) H. Cao and N. Kuboyama: *Bone* **46** (2010) 386–395.