Effect of the Introduction of a Carboxylic Acid Group onto the Surface of a Three-dimensional Porous Poly (lactic acid) Scaffold on Apatite Deposition

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Abstract

Scaffolds are important in engineering biomaterials for tissue and bone regeneration. In this study, a three-dimensional porous poly (lactic acid) (PLA) scaffold was fabricated using a method of salt leaching followed by freeze drying. A carboxylic acid group (COOH) was introduced onto all surfaces of a three-dimensional (3D) porous PLA scaffold (PLA-COOH) to test whether this polar organic acid had an effect on apatite attachment and deposition in Hanks' balanced salt solution (HBSS, pH=7.4) compared to PLA alone. Water adsorption in phosphate-buffered saline (PBS) was also examined between PLA-COOH and PLA by varying immersion time to evaluate whether there is an effect of COOH on the rate and degree of degradation. In contrast to PLA scaffold alone that allowed for apatite deposition only on its surface, the PLA-COOH scaffold also allowed for apatite formation on the surface of the barrier walls within the pores of the scaffold. Although PBS immersion eventually degraded both porous PLA and PLA-COOH scaffolds, we did not observe any specific difference in the rate or degree of degradation between them. These results suggest that the COOH group introduced into the porous PLA scaffold is an improvement on the PLA biomaterial for effective and efficient bone regeneration.

Keywords :

PLA scaffold, biomaterials, hydrox-yapatite, Hanks' balanced salt solution, carboxylic acid group

Introduction

In the dental field, biodegradable polymers such as poly (lactic acid) (PLA), poly (glycolic acid) (PGA), or poly (lactic acid-glycolic acid) (PLGA) have been used as membranes to engineer guided tissue regeneration (GTR) or guided bone regeneration (GBR) (1-4). GTR or GBR membranes function to make space for bone regeneration by preventing the growth of fast-proliferating epithelial cells into the empty space of the missing bone or tissue.

In order to improve the biological activity of PLA, such as cell attachment, biocompatibility or osteoconductivity, Nagai *et al.* (5) and Fuse *et al.* (6) immobilized fibronectin—a cell-adhesive protein—on the surface of a PLA film by using the attachment method used by Cai *et al.* (7). They reported that fibronectin immobilization enhanced the cell attachment and proliferation on the surface of the PLA

film. The addition of fibronectin also affected the morphology of the cells attached to the PLA. These studies indicated that PLA could be modified by the addition of other groups or proteins to improve upon a desired effect of the scaffold material.

In the biomaterials field, it is common to assess the biological effect of biomaterials for regenerative medicine by testing the new systems in cell culture assays or by direct implantation into animals. However, an argument can be made that some preliminary experiments should be conducted first outside of the animals in order to better predict the biological effect of biomaterials and to protect the welfare of research animals. One such method is an immersion experiment using simulated body fluid (SBF) that has been shown to be valuable for predicting the bone formation ability of biomaterials.

Kokubo and colleagues published many studies

using SBF immersion experiments to test the bone adaptability of biomaterials (8, 9). They developed their own SBF that was prepared in such a way that the ion concentration and composition were almost equal to human blood plasma. They found that apatite precipitated onto the surface of biomaterials when these biomaterials were immersed in SBF (10-16). Hanawa (17) and Hanawa *et al.* (18) confirmed that apatite precipitated on a titanium surface after immersion in Hanks' balanced salt solution (HBSS) at pH=7.4 that did not contain other organic species. Thus, this study revealed the efficacy of using HBSS as an SBF. Supporting this, Hayakawa *et al.* (19) also reported apatite deposition on a phosphorylated bonding agent after immersion in HBSS.

On the basis of abovementioned reports, Kitahara (20) introduced a carboxylic acid group onto the surface of the PLA film by using sodium hydroxide (NaOH) solution and investigated apatite deposition onto the modified PLA surface. This study found that the introduction of carboxylic acid group on the PLA film surface hastened apatite attachment and was therefore more effective in inducing initial apatite deposition compared to the PLA film alone.

In this study, we tested whether we could extend

these previous findings to three-dimensional (3D) PLA biomaterials by introducing a carboxylic acid group (COOH) onto the 3D porous PLA scaffold (PLA-COOH) and determining whether this addition further increased apatite deposition. To evaluate this, we examined the apatite deposition on both the scaffold surface and within the pores of the 3D porous PLA-COOH as compared to 3D PLA scaffold alone. To evaluate whether the addition of COOH also affected the degradation of PLA, we also tested the effect of COOH on water adsorption in phosphate-buffered saline (PBS) between the 2 groups.

Materials and Methods

Preparation of 3D porous PLA scaffolds

The 3D porous PLA scaffolds were prepared by a method using salt-leaching followed by freeze-drying. One gram of PLA (Purasorb®, inherent viscosity: 2.53 dL/g, PURAC Biochem, Gorinchem, The Netherlands) was dissolved in 10 mL of dioxane. Sodium chloride (NaCl) particles were incorporated into 10% wt PLA/dioxane solution. In this reagent, the ratio of NaCl to 10% PLA/dioxane solution was 1:1 (5, 6). Next, the mixture was poured into the steel mold with a diameter of 5 cm, frozen using

Fig. 1. Scheme for surface modification of carboxylic acid group introduction onto the surface of a 3D porous PLA scaffold.

liquid nitrogen for 24 hours, and then lyophilized under vacuum to remove dioxane. Thereafter, the 3D porous PLA scaffold was prepared after being washed, rinsed with distilled water for 24 hours, and then dried using a desiccator. The size of the specimen obtained by this method of salt leaching and freeze drying was 4.5 cm in diameter and 0.4 cm in thickness.

Surface modification of 3D porous PLA scaffolds

The scheme for surface modification to introduce a carboxylic acid group onto the surface of the 3D porous PLA scaffold (PLA-COOH) is shown in Fig. 1. In accordance with Nagai's report (5), PLA scaffold was immersed in 0.2 N NaOH solution at 20 °C for 1 hour to hydrolyze the surface of the porous PLA scaffold. After this, the PLA-COOH scaffold was prepared after being rinsed with double-distilled water, 0.1 N HCl, and again with double-distilled water. The product was then dried and stored in a desiccator until use.

HBSS immersion test

The 3D porous PLA and PLA-COOH scaffolds were immersed in 30 mL HBSS (Table 1) (17) for 3, 7, 14, and 28 days in a sealed polystyrene bottle at room temperature. The solution and bottle were exchanged every day to maintain the specimens in fresh solution. At the end of storage period, the specimens were rinsed with distilled water to remove HBSS and then immediately dried in a desiccator.

To monitor apatite deposition onto the surface of porous PLA and PLA-COOH scaffolds as well as

onto the barrier walls within the pores, the surface and cross-section of PLA and PLA-COOH scaffolds were observed in fine detail using a scanning electron microscope (SEM, S-2700, Hitachi, Tokyo, Japan) at an accelerating voltage of 15 kV. The specimens were coated with gold and palladium before SEM observation. Cross-sections were obtained by vertically cutting the scaffolds using a razor blade (Microtome Blade C35, pfm medical, Germany).

Water adsorption in phosphate-buffered saline (PBS)

Both porous PLA and PLA-COOH scaffolds were immersed in 30 mL of PBS (pH=7.4) for 8 weeks in a glass vial at 20 °C. The solution was exchanged every day with the same volume of PBS. The weight of the specimens before and after immersion in PBS was measured to obtain the weight change of each sample. After wiping the sample surface with a paper towel (Kimwipe, S-200, Nippon Paper Crecia Co., Ltd. Tokyo, Japan) to remove water deposited on its surface, porous PLA and PLA-COOH scaffolds were dried at room temperature for 10 minutes, and the weight of each sample was then determined for each of the 4 specimens in each group. Water adsorption was calculated by dividing the difference in the weight of each sample before and after immersion by the weight of the respective sample before immersion. From this, the average water adsorption of each porous scaffold and accompanying standard deviation (SD) were calculated for each immersion time. The results were analyzed by Student's t-test, and significance was determined by achieving a value of $p < 0.05$.

Results

Effect of the addition of the COOH group to 3D porous PLA scaffolds on apatite deposition onto the surface of PLA and the barrier wall of pores

To confirm previous observations by Kitahara (20) and to determine whether the addition of a carboxylic acid group into PLA can also alter the apatite deposition capacity in 3D porous PLA, we created PLA and PLA-COOH scaffolds and compared their

Table 1. Concentration of ions in Hanks' balanced salt solution

Ion	Concentration (mol/L)
Na ⁺	1.42×10^{-1}
K ⁺	5.81×10^{-3}
Mg ²⁺	8.11×10^{-4}
Ca ²⁺	1.26×10^{-3}
Cl ⁻	1.45×10^{-1}
HPO ₄ ²⁻	7.78×10^{-4}
SO ₄ ²⁻	8.11×10^{-4}
HCO ₃ ⁻	4.17×10^{-3}

Fig. 2. The appearance of the 3D porous PLA scaffold and the cross-section view within porous PLA scaffold.
(a. Macroscopic appearance of 3D porous PLA scaffold)
(b. SEM view of the cross-section of 3D porous PLA scaffold, bar = 100 μ m)

respective abilities to attract apatite deposition after immersion in HBSS. Fig. 2 shows the macroscopic appearance of 3D porous PLA scaffold (Fig. 2A), which was prepared by using the salt-leaching and freeze-drying method, as well as the SEM view of its cross-section (Fig. 2B) prior to immersion in HBSS. The pore size created within scaffold was approximately 15-30 μ m in diameter.

After immersion in HBSS for 3, 7, 14 and 28 days, we found that higher levels of apatite precipitate

Fig. 3. SEM views of the surfaces of 3D porous PLA and PLA-COOH scaffolds after immersion in HBSS for 3, 7, 14 and 28 days.
Higher levels of apatite precipitate were observed in the 3D porous PLA-COOH scaffold surface than in the PLA scaffold surface after 3 days, where we observed only a few apatite deposits. With prolonged immersion of both scaffolds over time, the amount of precipitates increased.

were observed in the 3D porous PLA-COOH scaffold surface than in the PLA scaffold surface after 3 days, where we observed only a few apatite deposits (Fig. 3). With prolonged immersion of both scaffolds over time, the amount of precipitates increased. From this experiment, our data indicate that the PLA-COOH scaffold hastened the initial apatite attachment and allowed for the deposition of greater amounts of apatite onto the surface more effectively than the PLA scaffold did. Indeed, there is a clear difference between porous PLA and PLA-COOH scaffold, especially as observed at the early stage after immersion.

At 28 days after HBSS immersion, we evaluated the apatite deposits at the surface periphery and central cross-section of the 3D porous PLA and PLA-COOH scaffolds by SEM and present this data in Fig. 4. While precipitates were observed on the surface periphery of the control porous PLA scaffold, they were not observed on the barrier walls within the pores as evaluated by the central cross-section cut of the porous PLA scaffold. In contrast, the

precipitates were observed on both the barrier walls within the pores as well as on the surface periphery of the 3D porous PLA-COOH scaffold. This indicates that the addition of the COOH group onto the PLA scaffold increases the ability of PLA to attract apatite deposition on the surface of PLA and also allows for apatite deposition within the pores themselves, which is not seen in the control 3D porous PLA scaffold.

Effect of the addition of the COOH group to 3D porous PLA scaffolds on its rate and degree of degradation

To determine whether modification of the PLA scaffold by addition of COOH affected the rate and degree of scaffold degradation over time, we tested the water adsorption ability of PLA and PLA-COOH over time. Fig. 5 shows changes in water adsorption of porous PLA and PLA-COOH scaffolds as a function of immersion time in PBS. Both PLA and PLA-COOH scaffolds showed increases in water adsorp-

Fig. 4. SEM views of the surface periphery and central cross-section of porous PLA and PLA-COOH scaffolds after immersion in HBSS for 28 days.

While precipitates were observed on the surface periphery of the control porous PLA scaffold, they were not observed on the barrier walls within the pores as evaluated by the central cross-section cut of the porous PLA scaffold. In contrast, the precipitates were observed on both the barrier walls within the pores as well as on the surface periphery of the 3D porous PLA-COOH scaffold.

Fig. 5. Changes in water adsorption of porous PLA and PLA-COOH scaffolds as a function of immersion time. Both PLA and PLA-COOH scaffolds showed increases in water adsorption until 28 days. However, the water adsorption decreased over time during the 8 week experiment. Significant differences in water adsorption were not observed between the porous PLA and PLA-COOH scaffolds in each immersion time ($p > 0.05$).

tion until 28 days. However, the water adsorption decreased over time during the 8 week experiment. Significant differences in water adsorption were not observed between the porous PLA and PLA-COOH scaffolds in each immersion time ($p > 0.05$). This indicates that addition of the COOH group to PLA scaffolding did not alter its rate and degree of degradation.

Discussion

In the present study, we introduced a carboxylic acid group onto the surface of the 3D porous PLA scaffold and examined its effect on apatite deposition onto the surface and within pores of the scaffolds as well as on the rate and degree of scaffold degradation.

To evaluate in vitro bioactivity of the 3D porous PLA scaffolds designed here, such as osteoconductivity, HBSS was utilized as a simulated body fluid in this study. We found that the introduction of a carboxylic acid group onto the surface of porous PLA scaffold allowed for apatite deposition not only on the surface of the scaffold but also on the barrier walls of the pores within the scaffold. These results suggest that the 3D porous PLA scaffold has an interconnected pore structure and the barrier wall

surface within each of these pores is also able to be modified with the carboxylic acid group as the same as the surface of PLA scaffold. However, the mechanism of how this happens is still unclear. It is speculated that positively charged calcium ions bind easily to the PLA-COOH surface through the interaction of calcium ions and carboxylate anion on the PLA-COOH surface. Afterwards, negatively charged phosphate ions can adsorb onto the PLA-COOH bound calcium ion. Indeed, it has been observed that new globule crystals were piled up on earlier precipitates on both PLA and PLA-COOH scaffolds. (18, 19). In contrast, the PLA scaffold only allowed for the apatite formation on its surface in our study. This is likely due to the surface characteristic of the modified PLA scaffold affecting the initial apatite attachment (20).

Both porous PLA and PLA-COOH scaffolds exhibited a maximum water adsorption at an immersion time of 28 days. Further increases in immersion time led to decreases in the water adsorption by both types of scaffolds. The observed decreases in water adsorption were probably due to the degradation of both porous PLA and PLA-COOH scaffolds (20). Kitahara (20) reported that the introduction of carboxylic acid onto the surface of the PLA film caused a more rapid and increased the rate and degree of degradation of the PLA film as compared to the unmodified PLA when immersed in PBS solution. This was due to the carboxylic acid-induced hydrolysis of the PLA as an autocatalysis reaction (21). However, our result differed from the result reported by Kitahara (20) because we did not find differences in the rate or degree of degradation between PLA and PLA-COOH scaffolds. This difference in results was likely due to differences between the PLA types used in the 2 studies, such as in the morphological structure of the specimens, including thickness, shape, and the number and size of pores.

In spite of the limitations in the present study, the following conclusions can be established from our results. The introduction of carboxylic acid group onto the surface of the 3D porous PLA scaffold hastened the initial apatite deposition and allowed

for deposition not only on the surface of the scaffold but also on the surface of the barrier walls within the pores of the scaffold. The results presented here suggest that carboxylic acid modification of porous PLA scaffolds can be used to create effective and efficient bone regeneration biomaterials.

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References

- Huang HH, He CL, Wang HS, Mo XM: Preparation of core-shell biodegradable microfibers for long-term drug delivery. *J Biomed Mater Res Part A*, 90: 1243-1251, 2009.
- Hutmacher D, Hürzeler MB, Schliephake H: A review of material properties of biodegradable and bioresorbable polymers and devices for GTR and GBR applications. *Int J Oral Maxillofac Implants*, 11: 667-678, 1996.
- AlGhamdi AS, Ciancio SG: Guided tissue regeneration membranes for periodontal regeneration- A literature review. *J Int Acad Periodonto*, 11: 226-231, 2009.
- Fujihara K, Kotaki M, Ramakrishna S: Guided bone regeneration membrane made of polycaprolactone/calcium carbonate composite nano-fiber. *Biomaterials*, 26: 4139-4147, 2005.
- Nagai M, Hayakawa T, Makimura M: Fibronectin immobilization using water-soluble carbodiimide on poly-L-lactic acid for enhancing initial fibroblast attachment. *J Biomater Appl*, 21: 33-47, 2006.
- Fuse M, Hayakawa T, Fukumoto M, Makimura M: Effect of fibronectin immobilization on poly-L-lactic acid films for the adhesion and proliferation of mouse osteoblast-like cells. *Nano Biomedicine*, 2: 93-102, 2010.
- Cai K, Yao K, Lin S, Yang Z, Li X, Xie H, Qing T, Gao L: Poly (D, L-lactic acid. surfaces modified by silk fibroin: effects on the culture of osteoblast in vitro. *Biomaterials*, 23: 1153-1160, 2002.
- Kokubo T, Ito S, Huang ZT, Hayashi T, Sakka S: Ca, P-rich layer formed on high-strength bioactive glass-ceramic A-W. *J Biomed Mater Res*, 24: 331-343, 1990.
- Kokubo T, Takadama H: How useful is SBF in predicting *in vivo* bone bioactivity? *J Biomaterials*, 27: 2907-2915, 2006.
- Tanahashi M, Yao T, Kokubo T, Minoda M, Miyamoto T, Nakamura T, Yamamuro T: Apatite coated on organic polymers by biomimetic process: Improvement in its adhesion to substrate by glow-discharge treatment. *J Biomed Mater Res*, 29: 349-357, 1995.
- Takadama H, Kim HM, Kokubo T, Nakamura T: TEM-EDX study of mechanism of bonelike apatite formation on bioactive titanium metal in simulated body fluid. *J Biomed Mater Res*, 57: 441-448, 2001.
- Kim HM, Kishimoto K, Miyaji F, Kokubo T, Yao T, Suetsugu Y, Tanaka J, Nakamura T: Composition and structure of apatite formed on organic polymer in simulated body fluid with a high content of carbonate ion. *J Mater Sci Mater Med*, 11: 421-426, 2000.
- Kim HM, Himeno T, Kawashita M, Lee JH, Kokubo T, Nakamura T: Surface potential change in bioactive titanium metal during the process of apatite formation in simulated body fluid. *J Biomed Mater Res*, 67A: 1305-1309, 2003.
- Kim HM, Himeno T, Kawashita M, Kokubo T, Nakamura T: The mechanism of biomineralization of bone-like apatite on synthetic hydroxyapatite: an *in vitro* assessment. *J R Soc Interface*, 1: 17-22, 2004.
- Kim HM, Himeno T, Kokubo T, Nakamura T: Process and kinetics of bonelike apatite formation on sintered hydroxyapatite in a simulated body fluid. *Biomaterials*, 26: 4366-4373, 2005.
- Hashimoto M, Takadama H, Mizuno M, Kokubo T: Mechanical properties and apatite forming ability of TiO₂ nanoparticles/high density polyethylene composite, Effect of filler content. *J Mater Sci Mater Med*, 18: 661-668, 2007.
- Hanawa T: Titanium and its oxide film: A substrate for formation of apatite. *In*: Davies JE. Ed. *The Bone-Biomaterial Interface*: 49-61, 1991.
- Hanawa T, Ota M: Calcium phosphate naturally formed on titanium in electrolyte solution. *Biomaterials*, 12: 767-774, 1991.
- Hayakawa T, Yoshinari M, Sakae T, Nemoto K: Calcium phosphate formation on the phosphorylated dental bonding agent in electrolyte solution. *J Oral*

- Rehabil, 31 : 67-73, 2004.
20. Kitahara S : Effect of surface hydrolysis of PLA film on apatite formation *in vitro*. J Oral Tissue Engin, 6 : 77-87, 2008.
21. Pitt CG, Gu ZW : Modification of the rates of chain cleavage of poly (ϵ -caprolactone. and related polyesters in the solid state. J Controlled Release, 4 : 283-292, 1987.