Bone augmentation using a highly porous PLGA/β-TCP scaffold containing fibroblast growth factor-2.

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Running title
Bone conduction by FGF2/β-TCP scaffold.

Key words
β-tricalcium phosphate (β-TCP); co-poly lactic acid/glycolic acid (PLGA); fibroblast growth factor-2 (FGF2); bone tissue engineering.

Abstract
Background and objective: β-tricalcium phosphate (β-TCP), a bio-absorbable ceramic, facilitates bone conductivity. We constructed a highly porous three dimensional scaffold using β-TCP for bone tissue engineering and coated it with co-poly lactic acid/glycolic acid (PLGA) to improve the mechanical strength and biological performance. The aim of this study was to examine the effect of the implantation of the PLGA/β-TCP scaffold loaded with fibroblast growth factor-2 (FGF2) on bone augmentation.

Material and methods: The β-TCP scaffold was fabricated by the replica method using polyurethane foam, then coated with PLGA. The PLGA/β-TCP scaffold was characterized by SEM, TEM, XRD, compressive testing, cell culture, and a subcutaneous implant test. Subsequently, a bone forming test was performed using fifty two rats. The β-TCP scaffold, PLGA-coated scaffold, and β-TCP scaffold and PLGA-coated scaffolds loaded with FGF2, were implanted into rat cranial bone. Histological observations were made at 10 and 35 days post-surgery.
**Results:** SEM and TEM observations showed a thin PLGA layer on the β-TCP particles after coating. High porosity of the scaffold was exhibited after PLGA coating (> 90%), and the compressive strength of the PLGA/β-TCP scaffold was 6-fold greater than the non-coated scaffold. Good biocompatibility of the PLGA/β-TCP scaffold was found in the culture and implant tests. Histological samples obtained following implantation of PLGA/β-TCP scaffold loaded with FGF2 showed significant bone augmentation.

**Conclusion:** The PLGA coating improved the mechanical strength of β-TCP scaffolds while maintaining high porosity and tissue compatibility. PLGA/β-TCP scaffolds in combination with FGF2 are bioeffective for bone augmentation.

**Introduction**

Tissue engineering requires that the proliferation, migration and differentiation of regenerative cells be stimulated to reform functional tissue following surgery (1); therefore, three-dimensional scaffolds with various regenerative properties are being extensively studied. Various synthesized bioceramic materials such as calcium phosphate (2), hydroxyapatite (HA) (3), bioactive glass (4) and composites (5) in various forms have been used as filling materials and scaffolds for osseous defect repair. Of these, β-tricalcium phosphate (β-TCP) has high biocompatibility and osteoconductivity in vivo and is more bio-absorbable than HA or bioactive glass (6). The clinical effects of bone graft substitutes using β-TCP on bone repair and regeneration have been widely recognized in the orthopedic and dental fields (7, 8).

Regenerative scaffolds should induce rapid reconstructive tissue-ingrowth through wound healing. Up-regulation of the ingrowth of regenerative tissue and blood vessels into the scaffold is generally relevant to the morphology of the scaffold (9). β-TCP clinically applied as a bone graft material controls pore characteristics such as pore volume fraction, size and structure. Although lower porosity materials have the high physical strength required for applications in the body (10), these materials tend to occlude tissue-ingrowth because of their lower internal space (11). Furthermore, scaffold morphology is strongly associated with biodegradability. The bio-safe degradation of the scaffold influences tissue remodeling and the creation of functional organs and tissues (12). To address some of these issues, in the current study we created a β-TCP scaffold by the replica method using polyurethane foam (13). The scaffold contained interconnections between open-cells and larger inner spaces (porosity > 90%), thus promoting its integration into the surrounding tissue. However, there was a practical problem: the high porosity resulted in lower mechanical strength, and the scaffold could not maintain its regenerative three dimensional structure. Previously, ceramics in combination with biodegradable plastics provided novel scaffolds with improved mechanical characteristics (14). For example, co-poly lactic acid/glycolic acid (PLGA) is a useful material with good plasticity and biocompatibility (15). Thus, a PLGA coating should increase the strength of a
highly porous scaffold and aid the invasion of cells into the regenerative space during the early stages of healing.

Fibroblast growth factor-2 (FGF2) stimulates cell viability associated with wound healing (16). A scaffold containing FGF2 promoted rapid tissue ingrowth in the inner spaces of the scaffold (17). FGF2 also regulates the proliferation and differentiation of osteogenic cells (18). Recently, FGF2 in combination with bioceramics, including β-TCP, enhanced bone formation in animal studies (18, 19). Furthermore, a successful clinical trial of FGF2 was performed in patients with bone defects caused by periodontitis (20). These findings lead us to hypothesize that FGF2 application might accelerate bone ingrowth into a highly porous β-TCP scaffold coated with PLGA, resulting in bone repair and bone creation. However, to date there have been no histological examinations of implanted β-TCP scaffolds coated with PLGA and impregnated with FGF2. Accordingly, the aim of the present study was to examine the effect of implantation of a highly porous PLGA/β-TCP scaffold loaded with FGF2 on bone augmentation in rats.

Materials and Methods

Preparation of the PLGA/β-TCP scaffold

A β-TCP slurry was prepared from β-TCP powder (average particle size: 2.3 µm) provided by Tomita Pharmaceuticals (Naruto, Japan) and carboxymethylcellulose (Daicel Corporation, Tokyo, Japan) as the binder. Polyurethane foam with a 0.5 mm cell size (MF-20, Inoac Corporation, Nagoya, Japan) was cut and immersed in the β-TCP slurry. After drying, the foam with β-TCP was sintered in a furnace (1150°C, 1 h). β-TCP scaffold was prepared with a 0.4 mm open cell size (Fig. 1-A). 50:50 PLGA powder (PLGA5005, Wako Pure Chemical Industries, Osaka, Japan) was dissolved in acetone. Several β-TCP scaffolds were dipped into PLGA solution (20 wt%) and dried at 100°C for 24 h (Fig. 1-A).

Characterization of the PLGA/β-TCP scaffold

The scaffold was analyzed using a scanning electron microscope (SEM, S-4000, Hitachi, Tokyo, Japan) at an accelerating voltage of 10 kV after coating with a thin layer of Pt-PD, and with a transmission electron microscope (TEM, HD-2000, Hitachi, Tokyo, Japan) coupled with an energy dispersive X-ray spectroscopy unit (EDS) at 200 kV acceleration voltage. Pure β-TCP powder, β-TCP scaffold and PLGA-coated scaffold were characterized using X-ray diffraction (XRD, RINT2000, Rigaku, Tokyo, Japan). Cu Kα radiation at 40 kV and 40 mA was used. Diffractograms were obtained from 2θ = 10° to 90° with an increment of 0.02° at a scanning speed of 4°/minute. A compression test on the scaffold was performed using a universal testing machine (AG-X, Shimadzu, Kyoto, Japan). The cross-head loading speed was set at 1 mm/min. The porosity of the scaffold was calculated according to the following equation: porosity = 100 × (1−ρ1 / ρ2), where ρ1
Biocompatibility test using the PLGA/β-TCP scaffold.

In order to evaluate cytocompatibility, each scaffold was seeded with $1 \times 10^4$ mouse osteoblastic MC3T3-E1 cells and cultured in humidified 5% CO$_2$ at 37°C using medium (MEM alpha-GlutaMAX-I, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Qualified, Life Technologies) and 1% antibiotics (Pen Strep, Life Technologies). After 24 h culture, samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min, then rinsed in cacodylate buffer solution. The scaffold was dehydrated in increasing concentrations of ethanol and analyzed by SEM. Each scaffold was implanted in the subcutaneous tissue of rats. The experimental protocol followed the institutional animal use and care regulations of Hokkaido University (Animal Research Committee of Hokkaido University, Approval No. 10-42). Four 10-week-old male Wistar rats weighing 190 to 210 g were given general anesthesia by intraperitoneal injection of 0.6 ml/kg sodium pentobarbital (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan), as well as a local injection of 2% lidocaine hydrochloride with 1:80,000 epinephrine (Xylocaine Cartridge for Dental Use, Dentsply-Sankin K.K., Tokyo, Japan). After a skin incision was made, scaffold (size $2 \times 2 \times 2$ mm) with or without PLGA coating was implanted into the subcutaneous tissue of the back of the rats. Skin flaps were sutured (Softretch 4-0, GC, Tokyo, Japan) and tetracycline hydrochloride ointment (Achromycin Ointment, POLA Pharma, Tokyo, Japan) was applied to the wound. Ten days postsurgery, the rats were euthanized using an overdose of sodium pentobarbital (2.0 ml/kg). Implants were excised with the surrounding tissues, fixed in 10% buffered formalin, decalcified in 10% EDTA, and embedded in paraffin according to standard procedures. Six-micrometer-thick sections were prepared and stained with hematoxylin-eosin (HE). Sections were examined using light microscopy.

FGF2 implant preparation

FGF2 (Fiblast spray 500, Kaken Pharmaceutical, Tokyo, Japan) was diluted with distilled water (Otsuka distilled water, Otsuka Pharmaceutical, Tokyo, Japan) to produce a stock solution of 0.5 µg/µl, as described previously (18). The FGF2-loading groups (pure β-TCP and PLGA/β-TCP scaffolds (size $6 \times 6 \times 3$ mm)) received 100 µl FGF2 solution (loading dose: 50 µg) under vacuum. For the other groups, each material was immersed into distilled water.

Surgical procedure for bone forming test

Fifty two Wistar rats were given general anesthesia by intraperitoneal injection of sodium pentobarbital, as well as a local injection of 2% lidocaine hydrochloride with 1:80,000 epinephrine. After a skin incision was made, a flap was made in the scalp. Decortication of a 4 mm$^2$ area was
performed in front of the coronal suture in the cranial bone using a rotating round bur under water irrigation. Subsequently, one of four types of sample was placed on the cranial bone with decortication (Fig. 1-B): β-TCP scaffold, PLGA/β-TCP scaffold, and each material loaded with FGF2. As a control, no implantation was performed. Skin flaps were sutured and tetracycline hydrochloride ointment was applied to the wound. Rats were euthanized 10 or 35 days after surgery using an overdose of sodium pentobarbital and specimens were collected from the wound. Six µm sections located every 300 µm, including the cranial bone and surrounding soft tissue, were prepared. Sections were stained with HE and Masson’s trichrome and examined using light microscopy. Three sections were taken for histomorphometric measurements from each scaffold. The height of newly formed bone and residual scaffold, and the number of blood vessels and giant cells (Fig. 1-C), were measured in each stained section collected 35 days post-surgery using a software package (Image J 1.41, National Institute of Health, Bethesda, MD, USA).

Statistical analysis
The means and standard deviation of each parameter were calculated for each group. Statistical analysis was performed using the Mann-Whitney U test for porosity and compressive strength, and Bonferroni’s multiple comparison for each histometric measurement. P values < 0.05 were considered statistically significant. All statistical procedures were performed using a software package (DR. SPSS 11.0, SPSS Japan, Tokyo, Japan).

Results
Characterization of the PLGA/β-TCP scaffold
The PLGA/β-TCP scaffold showed a reticulated open-cell structure, as did the uncoated scaffold (Fig. 2-A, E). The interconnected spaces of the scaffold were preserved following the PLGA coating process. SEM observations of cross-sections of the strut revealed a tube-shaped structure (Fig. 2-B, F, defect size: 50 µm). A thin layer of PLGA was deposited onto the sintered β-TCP (Fig. 2-C, D). Several micropores with a diameter of approx. 2 µm were observed on the scaffold (Fig. 2-C, G). These tube-shaped spaces and micropores resulted from the dissolution and subsequent combustion of the polyurethane foam (Fig. 2-C). TEM-EDS analysis of scaffold surfaces containing PLGA showed a higher content of carbon compared to scaffolds lacking PLGA (Fig. 3). XRD pattern analyses detected only diffraction peaks belonging to pure β-TCP from each β-TCP scaffold (Fig. 4). The porosity of each scaffold was calculated to be > 90%, indicating that there was no significant difference between the porosity of β-TCP scaffolds and PLGA-coated scaffolds (Table 1). The compression strength of the PLGA/β-TCP scaffold was about 6-fold greater than that of the uncoated scaffold, a difference which is statistically significant (Table 1).
**Biocompatibility of the PLGA/β-TCP scaffold.**

MC3T3-E1 cells attached to and spread on each scaffold (Fig. 5-A, B). In addition, we rarely found inflammatory cells around the scaffold in the subcutaneous implant test, suggesting that both scaffolds possess good biocompatibility. Scaffolds frequently were subjected to tissue-ingrowth and resorption by giant cells, regardless of the presence or absence of a PLGA coating (Fig. 5-C, D). These results show that the PLGA/β-TCP scaffold holds promise for biomedical applications.

**Evaluation of bone forming effects**

Histological specimens retrieved 10 days following β-TCP and PLGA/β-TCP scaffold implants showed tissue ingrowth limited to the periphery of the scaffold. Bone induction was rarely found on the cranial bone (Fig. 6-A, B, C, D). In contrast, implants using the FGF2-loaded scaffold showed active bone formation continuous with the pre-existing bone at the decortication area of the cranial bone. FGF2-loading stimulated woven trabecular bone formation (Fig. 6-E, F, G, H).

At 35 days post-implant, bone augmentation was promoted by all the β-TCP scaffold implants (Fig. 7). Bone height in groups receiving the β-TCP and PLGA/β-TCP scaffold was 2- and 4-fold greater than that in the control (no implantation), respectively (Fig. 8-A). β-TCP scaffold without the PLGA coating was frequently compressed on the cranial bone (Fig. 7-A). PLGA coated scaffold was more effective in stimulating bone formation than uncoated scaffold (Fig. 7-C). Residual β-TCP was encapsulated by cell-rich connective tissue, including giant cells. The strut of the β-TCP scaffold showed tissue ingrowth, indicating that connective tissue and bone-like tissue invaded the β-TCP block interior (Fig. 7-B, D).

Considerable bone formation was clearly detectable in the FGF2-loaded scaffold (Fig. 7-E, F), and bone augmentation was maximally accelerated by implantation of PLGA/β-TCP scaffold in combination with FGF2 (Fig. 7-G, H). Bone height in FGF2-loaded PLGA/β-TCP scaffold was 6-fold greater than that in the control (Fig. 8-A). The open cell structure of the scaffold was adequately maintained and occupied with ingrowth tissue, allowing sufficient inner regenerative space for further bone augmentation. The height of the PLGA/β-TCP scaffold with FGF2 was significantly greater than that of the other scaffolds (Fig. 8B). Blood vessel formation, in relation to wound healing, was frequently observed in the presence of FGF2 (Fig. 7-I, 8-C). Moreover, giant cells were frequently observed in the FGF group (Fig. 7-I, 8-D). In contrast, in the control, there was little evidence of bone augmentation (Fig. 7-J).

**Discussion**

The present study indicates that a PLGA coating on a β-TCP scaffold significantly facilitated cranial bone augmentation compared to pure β-TCP scaffold. Recent studies have reported that
β-TCP enhances bone conductivity following implantation (21, 22). Osteogenesis is associated with several parameters of the scaffold for bone tissue engineering, including density, interconnected structure and stability (23). In particular, the mechanical properties of regenerative scaffolds play a facilitative role in maintaining the space required for tissue reforming (13, 18). Our studies show that the PLGA/β-TCP scaffold has high compressive strength, suggesting that the stability of the β-TCP scaffold was reinforced by the PLGA coating and that the regenerative space was maintained. Furthermore, SEM images showed that the open cell structure of the scaffold was retained and was not filled with PLGA, so the PLGA/β-TCP scaffold maintains a significant inner tissue infiltration area. In contrast, pure β-TCP scaffold appeared to be compressed after implantation and gradually lost its cell-invading and regenerative inner space. Based on these results, the PLGA/β-TCP scaffold lead to bone augmentation following implantation.

In addition, our results show that FGF2-application to the scaffold consistently and significantly stimulated osseous conduction in the cranial bone, whether or not the PLGA coating was present on the scaffold. It has been reported that FGF2 promotes cell proliferation (24), vascularization (25) and wound healing (26). FGF2 enhances early wound healing in some organs. We also found dramatic effects of FGF2 on bone formation at 10 days. It seems likely that FGF2 was retained in the scaffold and exhibited various biological effects. In addition, at 35 days, PLGA/β-TCP scaffold impregnated with FGF2 significantly stimulated bone augmentation compared to the other scaffolds, indicating that the combination of FGF2 and PLGA/β-TCP scaffold is an effective osteoconductive scaffold. Our data suggest that PLGA/β-TCP scaffolds containing FGF2 exhibit a number of outstanding qualities. Several studies from other laboratories have also demonstrated that bone deposition on the surface of a β-TCP block is facilitated by loading the block with FGF2 (27, 28). However, our results demonstrated more frequent bone tissue invasion of the β-TCP block interior compared with previous studies. The evidence obtained in the present study suggests that bone tissue and remodeling of PLGA/β-TCP scaffold occurred simultaneously. Accordingly, the highly porous structure of the PLGA/β-TCP scaffold would be very beneficial for bone tissue engineering. Furthermore, we also speculate that FGF acted to up-regulate the maintenance of the regenerative space, as well as other cellular activities. The stability of the PLGA/β-TCP scaffold was likely reinforced by the application of FGF2: early construction of extracellular matrix and blood vessels, as well as cell invasion, occurred in the inner region of the scaffold by FGF2, maintaining the space for bone tissue augmentation.

To investigate biocompatibility, the PLGA/β-TCP scaffold was characterized by cell culture and subcutaneous implant tests. The results showed that osteoblastic E1 cells spread on the PLGA coating layer. Furthermore, PLGA did not stimulate an inflammatory response involving the accumulation of leukocytes and lymphocytes. PLGA is a biocompatible material frequently used in tissue engineering (29). PLGA degrades by hydrolysis of its ester linkages in the presence of water.
(30), but the acidic byproducts accumulating in a tissue engineering space would have adverse biological effects (31). However, we rarely observed adverse healing effects associated with PLGA coating in this study, suggesting that the PLGA coating had low cytotoxicity and a positive effect for bone tissue engineering. On the other hand, many investigators have demonstrated that implanted β-TCP is resorbed by macrophage-like giant cells without eliciting a cytotoxic response (32, 33). In the bone forming test, histological samples obtained 35 days after FGF2-loaded scaffold implantation frequently showed an increase in giant cells around the scaffold, suggesting active wound healing and remodeling. A previous study also revealed giant cells in FGF2-loaded collagen scaffold after implantation in rat oral mucosa (9). It has been demonstrated that β-TCP degradation promotes an osteogenic marker of osteoblastic cells, alkaline phosphatase activity, resulting from calcium ion release (11). Histological observation revealed that residual β-TCP was encapsulated by newly formed bone and cell-rich tissue, including giant cells, suggesting that resorption of β-TCP and osteogenesis occurred simultaneously. We hypothesize that these phenomena in relation to FGF2 application allowed the required environmental changes for osteogenesis to occur.

Conclusion
This study focused on characterizing PLGA/β-TCP scaffolds containing FGF2 for use in bone tissue engineering. The PLGA coating improves the mechanical strength of β-TCP scaffolds while maintaining high porosity and tissue compatibility. FGF2-loading of PLGA/β-TCP scaffolds strongly guided tissue ingrowth and cranial bone augmentation in rat. Therefore, a highly porous PLGA/β-TCP scaffold in combination with FGF2 is bioeffective for bone tissue engineering.

Acknowledgements
We thank Tomita Pharmaceutical Co., Ltd., for providing the β-TCP. This work was supported by JPSP KAKENHI Grant Number 22791916. The authors report no conflicts of interest related to this study.

References


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<th>Table 1. Compressive strength of the material (N = 6, mean ± SD)</th>
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<td><strong>β-TCP scaffold</strong></td>
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* Statistical difference compared to β-TCP scaffold
Figure 1. A) β-TCP scaffold (a) and PLGA-coated β-TCP scaffold (b). Scale bar represents 1 mm. B) Surgical procedure. The samples were placed on the cranial bone. C) Schematic drawing of the histomorphometric analysis. The frontal plane view indicates the following parameters: the height of newly formed bone (1) and the height of residual scaffold (2).
Figure 2. A) SEM image of the PLGA/β-TCP scaffold. B) Fracture site of the PLGA/β-TCP scaffold. The tube-shaped internal space was found in the strut of the scaffold. The space was not filled with PLGA. C) Higher magnification of the PLGA/β-TCP scaffold. Some micropores were open at the surface. D) Fracture surface of the PLGA-coated β-TCP particle (arrowheads). β-TCP particles were wrapped with a thin layer of PLGA (*). E) SEM micrograph of the β-TCP scaffold. F) Fracture region of the β-TCP scaffold. G) Higher magnification of the β-TCP scaffold. Micropores are observed at the surface of the strut. Scale bars represent 200 μm (A, E), 100 μm (B, F), 10 μm (C, G) and 1 μm (D).

Figure 3
TEM-EDS analysis of the surface of the PLGA/β-TCP scaffold (A, B, C, D) and pure β-TCP scaffold (E, F, G, H). TEM images (A, E), Ca-K map (B, F), C-K map (C, G), EDS spectra (D, H).
Scale bars represent 1 μm.

Figure 4
XRD patterns of pure β-TCP powder (a), β-TCP scaffold (b) and PLGA/β-TCP scaffold (c).

Figure 5
Biocompatibility of the scaffold. A) SEM images of β-TCP scaffold and B) PLGA/β-TCP scaffold. Cell spreading on the scaffolds is shown (white arrows). C) Histological specimens following
implantation of the β-TCP scaffold and D) the PLGA/β-TCP scaffold. Inflammatory cells were rare around the scaffolds (*). Connective tissue ingrowth and giant cells associated with material degradation (arrows) were frequently observed in the implants. Scale bar represents 10 μm (A, B) and 50 μm (C, D). Staining: HE (C, D).
Figure 6
Histological findings on cranial bone at 10 days. A) β-TCP scaffold. B) Higher magnification of the framed area in (A). C) PLGA/β-TCP scaffold. D) Higher magnification of the framed area in (C). Bone formation was rarely observed following implantation of β-TCP and PLGA/β-TCP scaffold. E) FGF2-loaded β-TCP scaffold. F) Higher magnification of the framed area in (E). G) FGF2-loaded PLGA/β-TCP scaffold. H) Higher magnification of the framed area in (G). Woven trabecular bone formation was stimulated by FGF2-loading. Abbreviations: NB, new bone. Scale bars represent 1 mm (A, C, E, G) and 50 μm (B, D, F, H). Staining: HE.
Figure 7
Histological findings on cranial bone at 35 days. A) β-TCP scaffold. Some new bone formation was evident and the scaffold was frequently compressed. B) Higher magnification of the framed area in (A). Ingrowth of bone-like tissue was found in the strut of the scaffold (*). C) PLGA/β-TCP scaffold. Bone augmentation was clearly promoted by implantation of this material. D) Higher magnification of the framed area in (C). Interconnected space of the strut of the scaffold (*) was filled with newly formed bone and connective tissue. E) FGF2-loaded β-TCP scaffold. Bone augmentation was accelerated by the implant. F) Higher magnification of the framed area in (E). Bone induction and degradation of scaffold (*) was frequently observed. G) PLGA/β-TCP scaffold with FGF2 application. Ingrowth of new bone as well as connective tissue was frequently found and the regenerative space was well-maintained. H) Higher magnification of the boxed area in (G). I) Resorption of the scaffold (*) by giant cells (arrows) was detected. Blood vessels were observed at peripheral areas of the implant. J) Control group. There was little evidence of bone augmentation. Abbreviations: NB, new bone. Scale bars represent 1 mm (A, C, E, G, J) and 50 μm (B, D, F, H, I). Staining: HE (A, B, C, E, F, G, H, I, J) and Masson’s trichrome (D).
Figure 8

Histomorphometric analysis at 35 days (N= 8, mean ± SD). A) Bone height. B) Residual scaffold height. C) Number of blood vessels. D) Number of giant cells. Ctrl, TCP, PLGA, TCP + FGF and PLGA + FGF indicate control, β-TCP scaffold, PLGA/β-TCP scaffold, FGF2-loaded β-TCP scaffold and FGF2-loaded PLGA/β-TCP scaffold, respectively. * P < 0.05.