Preparation and Evaluation of a Poly(Lactic-co-glycolic Acid) Membrane Containing β-TCP

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Introduction

Bone healing is one of the essential processes in the clinical field. Guided bone regeneration (GBR) is a treatment method for reconstructing neo-bone tissue by using a barrier membrane to prevent the ingrowth of fibrous tissues and protect against subsequent osteoconduction (1, 2). To ensure clinical success, the barrier membrane must be biocompatible, flexible, and mechanically strong (3).

Keywords: guided bone regeneration, electrospinning, poly(lactic-co-glycolic acid), β-tricalcium phosphate, alkaline phosphatase staining

Abstract

Purpose: Guided bone regeneration (GBR) is used in dental practices to increase the bone volume at bone defect sites for implant placement. The membranes used in GBR are non-absorbable or absorbable. Currently, the most various absorbable synthetic polymer membranes used could be detrimental to cells as they release acidic degradation products that lower the pH as the absorption progresses. Here, we developed a β-tricalcium phosphate (TCP)-containing poly(lactic-co-glycolic acid) (PLGA) membrane to enable cell proliferation and examined the mechanical properties and cell activity of membranes with variable β-TCP content.

Methods: The mechanical properties were evaluated by scanning electron microscopy (SEM), fiber diameter measurements, Fourier transform-infrared spectroscopy, and a tensile test. Cellular activity on the membrane was evaluated by measuring the water contact angles and performing alkaline phosphatase staining.

Results: SEM revealed that added β-TCP particles were embedded in the membranes, providing a seemingly rough surface on the inner side of the fibers. The water contact angles presented excellent wettability for PLGA/6 wt% β-TCP, PLGA/9 wt% β-TCP, and PLGA/12 wt% β-TCP. The tensile test showed excellent results for control, PLGA/3 wt% β-TCP, and PLGA/6 wt% β-TCP. Cell culture tests indicated that PLGA/9 wt% β-TCP and PLGA/12 wt% β-TCP positively affected the growth rate of cells cultured for an extended period.

Conclusion: When a PLGA/β-TCP membrane is prepared using the electrospinning method, the optimal β-TCP concentration is PLGA/3 and 6 wt% β-TCP.

Various materials have been used to fabricate GBR membranes, which are generally classified as non-absorbable or absorbable. Non-absorbable membranes exhibit excellent space-maintaining ability but are used only for specific indications because a second surgery is required to remove them, and there is a risk of infection caused by a high rate of membrane exposure.

Absorbable membranes are increasingly used in clinical practice, as many of their limitations have been addressed. These include their low space-maintaining ability via weak mechanical properties and their rapid
degradation and absorption. Recent studies have focused on improving the space-maintaining abilities and mechanical properties of absorbable membranes using different fabrication methods and synthetic biodegradable materials. Furthermore, among the several production methods that have been developed, electrospinning has gained attention in regenerative medicine because it can produce ultrafine fibers that physically mimic the natural bone extracellular matrix (ECM) at the nanoscale.

Among these methods, electrospinning is a cost-effective technique that can be used to synthesize continuous nanofibers. This approach can be used for soluble or fusible polymers alone, or the polymers can be modified with additives such as particles or enzymes to obtain the desired properties. The resulting ultrafine fibers exhibit a high surface area, tailorabile porosity in the range of the submicron scale to the nanoscale, and a high potential for surface functionalization. Additionally, electro fibers can be used in various biomedical applications such as in wound dressings, drug delivery, and tissue engineering scaffolds.

We prepared a membrane by electrospinning using a poly(lactic-co-glycolic acid) (PLGA) concentration of 20 wt%, as described by Lee et al. (2). The polymer PLGA is biodegradable and biocompatible and enhances the mechanical properties of materials. Hexafluoroisopropyl alcohol (HFIP) was used as a solvent to dissolve the polymer. Furthermore, the interaction of osteoblasts with the nano-sized surfaces of biomaterials is beneficial for the adhesion, proliferation, and differentiation of osteoblasts on the nano-sized rough surfaces of PLGA (8). However, PLGA degradation intrinsically decreases the pH of the regeneration niche, hampering the regeneration potential (9). To overcome this issue, β-tricalcium phosphate (β-TCP) was used to neutralize the acidic environment.

Numerous in vivo and in vitro assessments have shown that β-TCP has excellent biocompatibility and osteoconductivity, as well as supports the attachment, differentiation, and proliferation of relevant cells. Additionally, β-TCP exhibits a faster degradation rate than crystalline hydroxyapatite (HAp). Therefore, we developed a PLGA membrane containing β-TCP dissolved in HFIP to a PLGA concentration of 20 wt%. The β-TCP was added to improve cell activity.

However, the optimal amount of β-TCP that should be added to improve cell activity and maintain the mechanical properties of the membrane has not been examined. This study was conducted to add β-TCP to a slurry prepared at a PLGA concentration of 20 wt% and fabricate a membrane by electrospinning. We evaluated the mechanical properties of the membrane through fiber diameter, tensile strength, and Fourier transform-infrared spectroscopy (FT-IR) analyses with different amounts of β-TCP and evaluate cell activity based on the changes in cell behavior of the water contact angle and alkaline phosphatase (ALP) activity, to determine the range of β-TCP concentrations that are associated with the highest mechanical strength and cell activities.

### Materials and Methods

#### Materials

- PLGA 75/25 (inherent viscosity 0.55–0.75 dL/g) (DU-RECT Corporation, AL, USA), HFIP (Wako, Osaka, Japan), and β-TCP (β-TCP-100, Taihei Chem. Ind. Co., Ltd., Osaka, Japan) were used to fabricate the PLGA and PLGA/β-TCP membranes.
- The β-TCP powder was prepared in an Al2O3 container containing Al2O3 balls of one of two different sizes (ϕ=3 or 5 mm) for 18 h and using a planetary ball mill (P5/2, Fritsch Japan Co. Ltd., Kanagawa, Japan).

#### Preparation of polymer solutions

The PLGA solution was prepared by dissolving PLGA in 20 wt% HFIP. The PLGA/β-TCP polymer solutions with various compositions were prepared as listed in Table 1.

#### Electrospinning

The electrospinning set-up (IMC-1639, Imoto, Kyoto,
Japan) consisted of a high voltage power supply (Matsusada Precision, Inc., Shiga, Japan) and plastic syringe (Terumo Corp., Tokyo, Japan) fitted with a 21-G stainless steel needle (Terumo Corp.).

The flow rate of the polymer solution was maintained at 1.5 mL/h. A high voltage of 15 kV was applied at the tip of the needle, and a distance of 10 cm between the needle and ground electrode (d=9 cm; a stainless steel sheet on a drum whose rotation speed could be varied) was sustained throughout the process (12), the PLGA and PLGA/β-TCP solution was evaporated, and fibrous PLGA and PLGA/β-TCP were deposited on the ground electrode at 25°C. The obtained fibrous PLGA and PLGA/β-TCP was dried overnight at 25°C.

Physical characterization

1 Morphology

The detailed structures of respective PLGA/β-TCP membranes were imaged by scanning electron microscopy (SEM) (S-3400N; Hitachi, Tokyo, Japan) at an acceleration voltage of 15 kV. Before SEM observation, each sample was cut from a PLGA/β-TCP membrane (1×1 cm) and carbon vapor deposition was performed (13). The electrospinning fibers were analyzed manually by measuring the fiber diameters of 10 randomly selected fibers. Before undertaking these measurements, the contrast and threshold of each image were optimized to ensure that the fibers on the top layers were in focus. The analyses were performed using Adobe Photoshop 5.0 software (Adobe, San Jose, CA, USA).

2 Tensile test

The PLGA/β-TCP membranes were prepared with a test specimen punching blade No.6 (Kobunshi Keiki Co., Ltd., Kyoto, Japan). The tensile tests were performed on an EZ-Test (AGS-H; Shimadzu, Kyoto, Japan) at a cross-head speed of 10.0 mm/min. All experiments were conducted at 25°C (14). Each sample was measured six times, and the mean and standard deviation were determined.

3 Water contact angles

The water contact angle, which indicates the wettability of the material, was measured at 25 °C by Drop Master (DM300, Kyowa Interface Science Co., Ltd., Saitama, Japan). A PLGA/β-TCP membrane prepared at 1×1 cm was held at 25°C for 24 hours, and then attached to a glass slide for contact angle measurement (1). A single droplet of distilled water (2µl) was applied to the membrane surface and the contact angle was measured after 5 seconds. Seven measurements were taken at different locations, and the average value and standard deviation were determined.

4 FT-IR analysis

The PLGA/β-TCP membranes were characterized using a Nicolet iS 5 FT-IR (Thermo Fisher Scientific, Waltham, MA, USA). The infrared spectra of the samples were measured over a wavelength range of 4,000–500 cm⁻¹. All spectra were collected in the spectral range by the accumulation of 64 scans with a resolution of 4 cm⁻¹ (13).

Biological characterization

1 Cell culture and proliferation assay

MC3T3-E1 (mouse preosteoblasts, RIKEN BioResource Center, Tsukuba, Japan) were cultured in Dulbecco modified α-MEM containing 10% buffer solution (HEPES, Thermo Fisher Scientific) and 1% penicillin and streptomycin solution (Wako) at 37°C in 5% CO₂. The culture medium was changed every 3 days. For biological experiments, cultured cells were detached by trypsinization-EDTA (0.25% Trypsin-EDTA, Thermo Fisher Scientific), suspended in fresh culture media, and used in the experiments (15).

2 ALP staining

The PLGA/β-TCP membranes were first cleaned three times with PBS and then rinsed with isopropanol. The samples were then sterilized by UV irradiation for 20 min. Specimens measuring 3×3 cm were placed into the wells of a 6-well culture plate. MC3T3-E1 cells were then seeded into the wells at a density of 10,000 cells per well. The plate was incubated at 37°C for up to 1 week (3). After 1, 4, and 7 days of incubation, ALP staining was performed using the TRAP/ALP Stain Kit (Wako). After the staining, non-adherent cells were removed by washing three times with PBS. Next, adherent cells were collected by mild trypsinization and counted using a Coulter counter (Beckman Coulter, Tokyo, Japan). The experiments were repeated five times. The results are presented as the percentage of adherent cells.
Statistical analysis

All values are expressed as the mean ± standard deviation. Statistical comparisons were performed by analysis of variance, and the Bonferroni post hoc test was used to evaluate differences among groups. All statistical analyses were performed with PASW Statistics (Version 18.0, SPSS, Inc., Chicago, IL, USA). The results were considered to be statistically significant when $P < 0.05$. 

Results

Histological microscope observation

The results of SEM analysis are shown in Fig. 1. The nanofibers’ diameters were approximately uniform under SEM. The control groups appeared to be smooth and randomly arranged (Fig. 1a). The amount of β-TCP nanoparticles protruding from the fibers and exposed on the fiber surfaces (Fig. 1b–e) was exponentially proportional to the increase in β-TCP contents in composite fibers. Along with the increase in the β-TCP content, a lump of β-TCP particle was observed (Fig. 1d, e).

Fiber diameter

The results of the fiber diameter are shown in Fig. 2. Respective PLGA/β-TCP membranes were composed of uniform, randomly oriented fibers with diameters of 1.4-1.8 µm. The fiber diameter thickened in the following order: control (1.45 ± 0.26 µm), PLGA/3 wt% β-TCP (1.71 ± 0.26 µm), PLGA/6 wt% β-TCP (1.75 ± 0.58 µm), PLGA/9 wt% β-TCP (1.74 ± 0.52 µm), and PLGA/12 wt% β-TCP (1.83 ± 0.54 µm). There was no significant difference between the different PLGA/β-TCP membranes ($P > 0.05$).

Water contact angles

The results of the water contact angles are shown in Fig. 3. The water contact angle decreased in the following order: control (94.0 ± 9.29°), PLGA/3 wt% β-TCP (78.29 ± 3.13°), PLGA/6 wt% β-TCP (73.83 ± 6.02°), PLGA/9 wt% β-TCP (70.6 ± 4.16°), and PLGA/12 wt% β-TCP (64.5 ± 7.93°).

There was a significant difference between the control and all PLGA/β-TCP groups ($P < 0.05$). There was also a significant difference between PLGA/3 wt% β-TCP and PLGA/12 wt% β-TCP ($P < 0.05$).

Tensile test

The tensile test results are shown in Fig. 4. The tensile strengths were as follows from highest to lowest: PLGA/12 wt% β-TCP (9.14 ± 1.24 MPa), PLGA/9 wt% β-TCP (9.27 ± 0.84 MPa), PLGA/6 wt% β-TCP (10.53 ± 1.17 MPa), PLGA/3 wt% β-TCP (11.14 ± 0.85 MPa), and control (11.32 ± 0.97 MPa).

The mean difference between the PLGA/12 wt% β-TCP and control groups was 2.19 MPa; the tensile strength of the control group was 1.24-fold greater than that of the PLGA/12 wt% β-TCP membrane. There was a significant difference between the PLGA/12 wt% β-TCP and control membranes ($P < 0.05$).

The mean difference between the PLGA/9 wt% β
The mean difference between the PLGA/12 wt% β-TCP and control membranes (P < 0.05).

**FT-IR analysis**

The FT-IR spectra of the PLGA/β-TCP membranes showed that the nanofiber structure exhibited all peaks corresponding to PLGA (Fig. 5). The bands at 606 and 552 cm⁻¹ were ascribed to PO₄³⁻ from β-TCP, which appeared for PLGA/3 wt% β-TCP, PLGA/6 wt% β-TCP, PLGA/9 wt% β-TCP, and PLGA/12 wt% β-TCP, revealing that β-TCP was embedded in the PLGA matrix.

**ALP staining**

The measured numbers of adherent cells after 1, 4, and 7 days of culture on the different PLGA/β-TCP membranes are shown in Fig. 6 and 7.

After 1 day, there was no significant difference be-
between groups ($P > 0.05$).

After 4 days, PLGA/9 wt% $\beta$-TCP showed significantly more cultured cells than the control ($P < 0.05$).

After 7 days, 9 wt% $\beta$-TCP and PLGA/12 wt% $\beta$-TCP showed significantly more cultured cells than the control ($P < 0.05$).

**Discussion**

In this study, a slurry prepared using PLGA at a concentration of 20 wt% was added to $\beta$-TCP; a PLGA/$\beta$-TCP membrane was prepared using the electrospinning method; and the neutralizing effect on the acidic environment created by $\beta$-TCP was examined, as well as its effectiveness as a scaffold for cell proliferation.

The SEM findings showed that when $\beta$-TCP was added, the fibers expanded only in portions containing $\beta$-TCP granules, which increased the fiber diameter and surface area. Tensile testing showed that the addition of $\beta$-TCP led to a decrease in tensile strength to a lower magnitude as compared to $\beta$-TCP-free membranes. However, cell proliferation tests showed that when $\beta$-TCP was added, cell growth was significantly increased.

![Alkaline phosphatase staining of respective $\beta$-TCP concentrations after 1, 4 and 7 days (n=5).](image)

|          | After 1 day | After 4 days | After 7 days |
|----------|-------------|--------------|--------------|
| control  | ![Image](image) | ![Image](image) | ![Image](image) |
| PLGA/3wt% $\beta$-TCP | ![Image](image) | ![Image](image) | ![Image](image) |
| PLGA/6wt% $\beta$-TCP | ![Image](image) | ![Image](image) | ![Image](image) |
| PLGA/9wt% $\beta$-TCP | ![Image](image) | ![Image](image) | ![Image](image) |
| PLGA/12wt% $\beta$-TCP | ![Image](image) | ![Image](image) | ![Image](image) |
β-TCP reacts with body fluids, is degraded, resorbed, and replaces the adjacent bone. β-TCP has a greater solubility in body fluids than hydroxyapatite, and with time, β-TCP is degraded, resorbed, and replaced by bone. PLGA, which is a polymer, is prepared by changing the PLA-to-PGA ratio, and therefore, is characterized by the fact that its degradation rate and mechanical traits can be controlled. Besides, its use in vivo has been approved by the U.S. Food and Drug Administration (FDA), and its safety has been guaranteed. PLA, PCL, and E-PTFE have been used for the preparation of barrier films, but the membranes themselves do not maintain bioactive function. The following are required for bone formation: cells capable of differentiating into osteoblasts, biological factors controlling the cell growth and differentiation, and an osteoconductive bioabsorbable scaffold/matrix that promotes cell attachment, migration, and proliferation.

In our study, SEM findings showed no significant difference in the fiber diameters of membranes prepared with various amounts of β-TCP. In membranes prepared by adding β-TCP, β-TCP particles were found to be embedded in the membranes, and β-TCP particles contributed to rough fibers' surfaces. Yongcong et al. previously reported that electrospinning scaffold made from fibers with a diameter of 100 nm to 5 μm mimicked the shape of ECM (16), suggesting that the membrane can mimic the ECM morphology; besides, Smith et al. previously reported that coarse nanofiber surfaces prepared using apatite particles were able to promote the attachment and proliferation of osteogenic cells, as well as their differentiation into bone (17). Our study suggested that the addition of β-TCP particles to the membrane increased the membrane's surface area and led to an increase in the scaffold which supported cell attachment and growth.

The tensile strength of membranes made with PLGA/9 wt% β-TCP or PLGA/12 wt% β-TCP was significantly lower than that of the controls (P < 0.05). In addition, the findings also revealed that the amount of added β-TCP inversely correlated with tensile strength. This un-
expected correlation is in contrast with previous results by Ezati et al. According to this earlier work, β-TCP significantly worsens the scaffold’s mechanical properties (15). However, β-TCP did not form a chemical bond with PLGA and was made of small granules; therefore, it may be scattered through fibers, or it may weaken the fiber strength by adhering to the fiber surface. In conclusion, the β-TCP addition did not improve the mechanical strength of the fiber.

Our findings also showed that membranes prepared with the addition of β-TCP had a significantly smaller contact angle than that of the controls (P < 0.05). When a larger amount of β-TCP was added, β-TCP-like hydrophilic particles were exposed on the fiber surface, the surface became rough, and the membrane became more wettable. Arima et al. previously reported that surfaces with a moderate contact angle (40° to 70°) were capable of competitively adsorbing cell contact proteins, and could contribute to cell contact (18); this showed that the addition of β-TCP might supply a surface morphology that facilitates cell contact.

FT-IR findings confirmed that a peak representing PLGA at 865 cm\(^{-1}\) was present in each of the membranes, and bands at 606 and 552 cm\(^{-1}\) confirmed the presence of PO\(_4\)\(^{3-}\), which showed that the membranes contained β-TCP. This result was consistent with previous findings by Fei Lin et al. and suggested that β-TCP was unaffected by PLGA or HFIP (7).

Findings from cell culture tests showed that compared to the controls, cell growth was significantly higher in PLGA/9 wt% β-TCP after 4 days of cell culture and in PLGA/9 wt% β-TCP and PLGA/12 wt% β-TCP after 7 days of cell culture (P < 0.05). Our findings showed that PLGA/9 wt% β-TCP, as well as PLGA/12 wt% β-TCP, may have a positive effect on the growth rate of cells cultured for an extended period. In addition, Shim JH et al. previously reported that this positive effect could be due to the roughness of the surface of PCL/PLGA/β-TCP membranes (6) and that it may also affect cell morphology, cell adhesion, and cell growth. Our findings are also consistent with theirs, suggesting that the addition of β-TCP (PLGA/9 wt% β-TCP, PLGA/12 wt% β-TCP) has a positive effect on cell activity.

In conclusion, when a membrane is prepared with an addition of β-TCP using the electrospinning method, β-TCP is incorporated in the fibers or on the surface of fibers, resulting in a rough surface with a large surface area, which leads to an environment that facilitates cell adhesion and proliferation. However, membranes containing β-TCP at amounts of 9 wt% or 12 wt% (PLGA/9 wt% β-TCP and PLGA/12 wt% β-TCP) have a low mechanical strength; therefore, they cannot be used at sites that support heavy loads.

Therefore, PLGA/3 and 6 wt% β-TCP may be the optimal concentration to prepare PLGA/β-TCP membranes. Future studies to prepare membranes with higher mechanical strength and monitor the membrane over a more extended period are warranted. Moreover, we will study pH changes and perform immersion and in vivo experiments to further validate the clinical potential of PLGA membrane containing β-TCP.

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**Conflict of interest statement**

No conflict of interest exists.

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