Preparation of poly(γ-glutamic acid)/hydroxyapatite monolith via biomineralization for bone tissue engineering

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A hybrid monolith of poly(γ-glutamic acid) and hydroxyapatite (PGA/HAp monolith) was prepared via biomineralization and used as a macroporous cell scaffold in bone tissue engineering. The PGA monolith having a bimodal pore size distribution was used as a substrate to induce biomineralization. The PGA/HAp monolith was obtained by immersing the PGA monolith in simulated body fluid. Pretreatment with CaCl\textsubscript{2} enhanced the apatite-forming ability of the PGA monolith. Murine osteoblastic MC3T3-E1 cells efficiently attached and proliferated on the PGA/HAp monolith. MTT assay showed that both the PGA and PGA/HAp monolith did not have apparent cytotoxicity. Moreover, the PGA and PGA/HAp monoliths adsorbed bone morphogenetic protein-2 (BMP-2) by electrostatic interaction which was slowly released in the medium during cell culture. The PGA/HAp monolith enhanced BMP-2 induced alkaline phosphatase activity compared to the PGA monolith and a polystyrene culture plate. Thus, these PGA/HAp monoliths may have potential in bone tissue engineering.

**Keywords:** poly(γ-glutamic acid); monolith; hydroxyapatite; tissue engineering; biomineralization; thermally induced phase separation

1. Introduction

Poly(γ-glutamic acid) (PGA) is a hydrophilic, anionic, and biocompatible polypeptide produced by micro-organisms such as Bacillus subtilis.\textsuperscript{[1,2]} On the basis of these characteristics, PGA-based materials such as hydrogels and nanofibers have been designed for a wide range of applications, especially in the biomedical field.\textsuperscript{[3–6]} Of particular interest is PGA’s hydroxyapatite (HAp)-forming ability. HAp is one of the main inorganic components of the bone matrix, which confers mechanical strength to bone. Furthermore HAp promotes cellular attachment, proliferation, and osteogenic differentiation of osteoblasts.\textsuperscript{[7]} In natural bone, nucleation and crystal growth of HAp is induced by the glutamic acid-rich sequences present in type I collagen.\textsuperscript{[8]} In a similar manner, the glutamic acid residues of PGA are known to induce the formation of HAp.\textsuperscript{[9]}

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Due to the structural similarity with the natural bone matrix, hybrid materials composed of polymers and HAp are considered to be potential candidate scaffolds for bone tissue engineering.[10–14] Taking advantage of the apatite-forming ability of PGA, PGA/HAp hybrid materials have been prepared via biomineralization in simulated body fluid (SBF).[15,16] SBF is a solution with ion concentrations close to human blood plasma, that has been widely used to deposit bone-like apatite on the surface of organic substrates.[12] It has been reported that PGA hydrogels, PGA-coated collagen sponges, and silica–PGA hybrids were able to induce the nucleation of HAp in SBF.[15–17] In addition, several reports showed that the formation of HAp was further enhanced by incorporating Ca²⁺ ions in the scaffolds.[8,15] Although these PGA-based materials showed promise in bone regeneration, due to the hydrophilic nature of PGA, fabrication of well-defined structure often requires complicated procedures, which remains an issue in biological applications.

Apart from the chemical properties of the materials, there are several important morphological criteria in designing cell scaffolds. It is well documented that cell scaffolds with pores larger than several tens micrometer are required for three-dimensional cell proliferation. For example, pore size suitable for cell ingrowth is in the range of 100–400 μm for osteoblasts, 70–120 μm for chondrocytes, 38–150 μm for fibroblasts, and 63–150 μm for vascular smooth muscle cells.[11,18,19] Another requirement is efficient mass transfer which is essential to supply nutrition and oxygen to the cells. In addition, cell scaffolds should be able to incorporate bioactive molecules, such as growth factors, and release them in a controlled manner to stimulate cell proliferation and differentiation. For these reasons, it has been suggested that scaffolds with bimodal pore size distribution would be advantageous. Whereas large pores, generally >50 μm in diameter, offer space for cell ingrowth, smaller pores in the range of sub-hundred nanometer to several micrometer facilitate transport of nutrition and oxygen as well as supply high surface area for the incorporation of growth factors.[20,21]

The thermally induced phase separation (TIPS) technique is known as a simple method to prepare a well-defined monolith, a single-piece material having an interconnected porous network, from versatile polymers. Using this technique, our group have reported highly porous monoliths of poly(acrylonitrile), poly(bisphenol A carbonate), and poly(vinyl alcohol).[22–24] Recently, we prepared a PGA-based monolith by the TIPS technique followed by chemical cross-linking.[25] This monolith showed reversible deformability and thermal stability. Importantly, the monolith still had the unique properties of PGA such as hydrophilicity, metal-chelating ability, and pH responsiveness. By combining the TIPS technique with the well-known salt leaching technique we were able to fabricate a PGA monolith having homogeneously distributed submillimeter-sized cavities within an interconnected porous network.[26] This unique morphology together with the chemical and physical properties of PGA prompted us to investigate the potential use of this monolith as a cell scaffold in tissue engineering.

In this study, we report a hybrid monolith of PGA and HAp (PGA/HAp monolith) that may find application in bone tissue engineering. The PGA/HAp monolith was prepared via biomineralization of the PGA monolith in SBF. The effect of pretreatment with CaCl₂ aqueous solution on the HAp formation was evaluated. The behavior of murine osteoblastic cells (MC3T3-E1) on the monolith was also investigated. Furthermore, bone morphogenetic protein-2 (BMP-2) was adsorbed onto the monolith via electrostatic interaction and its biological effect on the osteogenic differentiation of MC3T3-E1 cells was assessed.
2. Experimental procedures

2.1. Materials

PGA (acid form, $M_w = 5 \times 10^3$ kDa) was provided by BioLeaders (Korea). Hexamethylene diisocyanate was purchased from Wako Pure Chemical Industries (Japan). Sodium chloride (+80 mesh particle size) and fluorescamine were purchased from Sigma-Aldrich. Recombinant human BMP-2 was purchased from R&D systems. Other reagents were commercially available and used without further purification. Water was freed from salt using a milliQ system and filtered (0.22 μm).

2.2. PGA monolith preparation

The PGA monolith was prepared as reported previously.[26] A typical procedure is as follows. PGA powder (450 mg) was dissolved in dimethyl sulfoxide/water/ethanol (9/1/20, 3 mL) at 80 °C. Sodium chloride particles (5.5 g) were then added to the solution and the mixture was cooled at 25 °C for 12 h. The resultant sodium chloride particles-incorporated PGA monolith was washed with acetone to remove the solvents and subsequently dried under vacuum. The as-prepared monolith (3.49 mmol monomer unit of PGA) was mixed with hexamethylene diisocyanate (1 mL, 5.89 mmol) in acetone (10 mL) and kept at 50 °C. After 6 h, water (300 μL) was added dropwise.[25,26] The monolith was washed with acetone and water to remove sodium chloride particles and unreacted cross-linker. Residual water was replaced with acetone and the monolith was dried in vacuo to yield the cross-linked PGA monolith.

2.3. Biomineralization on the PGA monolith

SBF was prepared by dissolving NaCl (8.035 g), NaHCO₃ (0.355 g), KCl (0.225 g), K₂HPO₄·3H₂O (0.231 g), MgCl₂·6H₂O (0.311 g), CaCl₂ (0.292 g), and Na₂SO₄ (0.072 g) into milliQ water (1 L). The pH was adjusted to 7.4 with tris-hydroxymethyl aminomethane (6.118 g) and HCl (1 M) aqueous solution at 37 °C.[12] Hydroxyapatite formation on the PGA monolith was carried out in SBF at 37 °C as reported by Kokubo.[12] The PGA monolith was cut into cubic shape (8 × 8 × 8 mm) and immersed in CaCl₂ aqueous solutions (50 mL) at various concentrations (0.01, 0.1, and 1 M) at 37 °C for 24 h. The monoliths treated with and without CaCl₂ were immersed in SBF (10 mL) at 37 °C for 1 and 7 d. SBF was replaced every day. Monoliths were washed with milliQ water and freeze-dried.

2.4. Characterization of the PGA/HAp monolith

Scanning electron microscopy (SEM) of the monoliths was done on a HITACHI S-3000N at an accelerated voltage of 15 kV. A thin gold film was sputtered on the samples before the images were collected. Energy-dispersive X-ray (EDX) spectra of the cross-section of the monoliths were recorded on a Hitachi Miniscope TM3000 with a Swift3000 equipment. X-ray diffraction (XRD) patterns were recorded on a Rigaku Multiflex diffractometer at 40 kV and 30 mA in continuous scanning degrees of 2θ min⁻¹. Fourier transform infrared spectra (FT-IR) were measured on a Thermo Scientific Nicolet iS5 with an attenuated total reflection accessory (iD5 ATR accessory). Ca and P concentrations were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES) using a Shimadzu ICP-7510 sequential plasma spectrometer.
2.5. **Culture of MC3T3-E1 cells**

MC3T3-E1 cells were obtained from the European Collection of Cell Cultures. Cells were cultured in α-minimum essential medium supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin in 75 cm² tissue culture flasks (Iwaki, Japan) at 37 °C under a humidified atmosphere with 5% CO₂. When cells reached 80–90% confluency, they were detached with 0.25% trypsin/EDTA and passaged at a ratio of 1:10.

2.6. **Cell attachment on the monoliths**

The PGA monolith and PGA/HAp monoliths were sterilized in 70% (v/v) ethanol/water and washed with sterile milliQ water to remove ethanol. A medical biopsy punch (6 mm; Kai Industries) and rat brain slicer (RBS-1, 1 mm; WPI) were used to cut the monoliths into small pieces (diameter 6 mm × thickness 1 mm).

Monolith pieces were placed in a 96-well culture plate and MC3T3-E1 cells in culture medium (200 μL) were seeded at a concentration of 1 × 10⁴ cells per well and cultured for 24 h. Cells on the monoliths were stained with the LIVE/DEAD Viability/Cytotoxicity Assay Kit (molecular probes, UK) and observed with an Olympus Fluoview FV1000-D confocal laser scanning fluorescence microscope.

2.7. **Preparation of rhodamine-labeled PGA monolith**

Rhodamine B functionalized with ethylene diamine (Rhodamine-NH₂) was prepared as reported.[27] A PGA monolith piece (5 mg) was placed in MES buffer (2 mL, 100 mM, pH 5) containing Rhodamine-NH₂ (0.4 mg). After adding EDC (74 mg) and sulfo-NHS (84 mg), the PGA monolith was incubated at 25 °C in the dark. After 20 h, the monolith piece was washed with phosphate buffer (pH 7.4) and water.

2.8. **Cell proliferation assay**

MC3T3-E1 cells were seeded on top of the monoliths (1 × 10⁴ cells per well) in culture medium (200 μL). Cell viability was evaluated by the 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. After the formazan crystals were solubilized in SDS (0.1 g/mL) in HCl (0.01 M), the absorbance at 570 nm was measured on a Tecan M200 Pro series well plate reader using 96-well polystyrene plates.

2.9. **BMP-2 loading**

The PGA monolith (3.2 mg, diameter 6 mm × thickness 1 mm) and PGA/HAp monoliths (4.3 mg, diameter 6 mm × thickness 1 mm) were immersed in Dulbecco’s PBS (200 μL) containing BMP-2 (0.5 and 1.0 μg) and kept at 4 °C for 3 d. The concentration of BMP-2 in the supernatant was quantified by enzyme-linked immune sorbent assay (ELISA) kit (R&D Systems, USA).

2.10. **Observation of BMP-2 adsorbed in the PGA/HAp monolith**

PGA/HAp monoliths with and without BMP-2 were immersed in PBS and 50 μL of fluorescamine (0.3 mg/mL in acetone) was added. After 5 min of incubation at RT, the monoliths were observed with an Olympus1X71 microscope.
2.11. Release of BMP-2 from the monolith in the presence of MC3T3-E1 cells

The PGA and PGA/HAp monoliths loaded with BMP-2 were placed in a 96-well culture plate. MC3T3-E1 cells (1 × 10^4 cells per well) in medium (200 μL) were seeded onto the monoliths and cultured for 12 d. The medium was replaced with fresh culture medium every third day. The concentration of BMP-2 in the culture medium was quantified by ELISA.

2.12. Alkaline phosphatase activity

MC3T3-E1 cells in medium (200 μL) were seeded onto the monoliths and cultured for 12 d replacing the culture medium every third day. As a control, the cells were cultured on a polystyrene plate for 12 d replacing the culture medium supplemented with/without BMP-2 (25 ng) every third day. Alkaline phosphatase (ALP) activity of MC3T3-E1 cells was measured using a LabAssay ALP kit (Wako, Japan) according to the manufacturer’s instructions. The monolith pieces containing MC3T3-E1 cells were washed with Dulbecco’s PBS (without Ca^{2+} and Mg^{2+}) and the cells were lysed in 0.25% Triton X-100 solution (0.2 mL) by shaking for 20 min at RT followed by one freeze–thaw cycle. The solution was collected and centrifuged. The supernatant (20 μL) was mixed with 6.7 mM p-nitrophenyl phosphate (pNPP) (100 μL) in carbonate buffer (0.1 M, pH 9.8) containing MgCl₂ (2.0 mM) and incubated at 37 °C for 15 min on a bench shaker. The reaction was stopped by adding NaOH (0.2 mM, 80 μL). Absorbance at 405 nm was measured on a plate reader. Total protein content was determined by the bicinchoninic acid (BCA) assay using QuantiPro BCA Assay kit (Sigma-Aldrich, USA).

2.13. Statistical analysis

All quantitative results were presented as the mean ± SD. The statistical difference between two groups was determined using the Student’s t-test.

3. Results and discussion

3.1. HAp formation on the PGA monolith in SBF

We first prepared the PGA monolith having an interconnected porous network (pore size: 200–800 nm) and cavities of 200–600 μm in dimension by combining the TIPS and salt leaching techniques.[26] We observed the morphology of the PGA monolith by SEM. Also, the PGA monolith was fluorescently labeled with rhodamine and observed by a confocal laser scanning fluorescence microscopy (CLSM). As shown in Figure 1, the monolith had submillimeter-sized cavities formed by salt leaching as well as a microporous network structure formed via phase separation.

The monolith was immersed in SBF to induce HAp formation. As shown in Figure 2(a), we observed that small spherical particles in the range of 0.1–0.6 μm in diameter were deposited within the porous network of the monolith after 7 d. After 30 d of incubation in SBF, larger spherical particles in the range of 1.0–3.0 μm were homogeneously distributed over the network structure (Figure S1). It is interesting to note that the monolith seemed to accelerate the crystal formation. Sugita et al. reported that only amorphous calcium phosphate was deposited and no spherical structure was found on the PGA hydrogel after 7 d of incubation in SBF.[15] Since the nucleation of HAp progresses through formation of amorphous calcium phosphate,[28] the crystal
forming process proceeded much faster on the monolith than hydrogel. Although the exact mechanism is not clear, it may be due to the high concentration of glutamic acid residues in the network structure where PGA molecules are bundled together.

To further accelerate apatite formation, we immersed the monoliths in aqueous CaCl$_2$ solutions at different concentrations before mineralization in SBF. It has been reported that the incorporation of CaCl$_2$ in the PGA matrix enhances the HAp formation by increasing the Ca$^{2+}$ concentration in the close vicinity of the substrate surface.[8,15] As confirmed by EDX, Ca was homogeneously distributed in the monolith (Figure 3). SEM images showed that the porous morphology of the monoliths was maintained after the CaCl$_2$ treatment (Figure S2). The surface roughness and skeleton size of the monolith seemed to increase slightly after 1 M CaCl$_2$ treatment, which may be due to the deposited CaCl$_2$ formed after drying the monolith in vacuo during SEM sample preparation. The CaCl$_2$-treated monoliths were immersed in SBF to induce HAp formation. Contrary to the untreated monolith, the CaCl$_2$-treated monoliths showed the formation of spherical crystals already after 1 d of incubation in SBF (Figure 2(b)–(d)). After 7 d, the apatite crystals had grown larger and were homogeneously distributed within the porous network of the monolith. Moreover, the concentration of the CaCl$_2$ solution showed a significant effect on the crystal growth. The size of the crystals increased as function of the CaCl$_2$ concentration; 0.2–1.0 μm for 0.01 M, 0.2–2.0 μm for 0.1 M, and 0.8–5.0 μm for 1 M.

To confirm the effect of mineralization on the monolith structure, we observed the monolith was treated with 1 M CaCl$_2$ and then immersed in SBF for 7 d at lower
magnification. As shown in Figure S3 in the Supplementary material, the submillimeter cavities were clearly visible and remained after SBF treatment. In addition, the micro-porous network structure was also partially visible showing that the micropores were not completely filled with the apatite crystals. Thus, it seems that even after mineralization, the monolith retained its unique structure having bimodal pore size distribution which would be important for cell infiltration and transport of nutrition and oxygen as well as incorporation of growth factors.
The mineralization process of the untreated and CaCl₂-treated monolith was compared by measuring the concentration of Ca²⁺ and PO₄³⁻ ions in the SBF solution. In this experiment, 1 M CaCl₂ was used to treat the monolith. Figure 4 shows the change of Ca and P concentration in the SBF as a function of time after the immersion of the monoliths. The Ca and P concentration in SBF gradually decreased in the presence of the untreated PGA monolith which is due to the consumption of Ca²⁺ and PO₄³⁻ ions during the HAp formation. In the case of the monolith treated with 1 M CaCl₂, the P concentration in SBF also decreased as a function of immersion time and the decrease was 60% higher than that for the untreated monoliths. This result suggests that CaCl₂ treatment enhances HAp formation. On the other hand, the Ca concentration increased within 1 d and remained constant for 1 week. This initial increase can be attributed to the release of incorporated Ca²⁺ ions from the pores of the monolith. Interestingly, the decrease of Ca concentration due to the HAp formation was not observed after the initial burst release of CaCl₂. This contradicting data may be due to the fact that not all Ca²⁺ ions were released into the SBF during the initial release and that

![Figure 4](image-url)

**Figure 4.** (a) Ca and (b) P concentrations in the supernatant as function of time after immersing the PGA monoliths in SBF (n = 3). *p < 0.05 vs. the non-treated PGA monoliths.
remaining Ca$^{2+}$ ions in the hydrated cross-linked monolith backbone were the calcium source for forming the HAp crystals.

To further characterize the PGA/HAp monolith and confirm HAp formation we used EDX, FT-IR, and XRD. As confirmed by EDX analysis of the cross-section of the monolith, both Ca and P peaks were detected on the 1 M CaCl$_2$-treated monolith after the immersion in SBF (Figure 5). The FT-IR spectrum of the PGA/HAp monolith showed bands due to phosphate anions which are typical of calcium phosphate. We observed two intense bands at 970–1170 and 560–600 cm$^{-1}$ due to the phosphate $\nu_3$ and $\nu_4$ vibrations and a weak peak at 950 cm$^{-1}$ due to the phosphate $\nu_1$ vibration (Figure 6).[8,29–31] Furthermore, XRD as shown in Figure 7 confirmed the presence of HAp crystals on the monolith by showing two peaks at 32° and 46° (2$\theta$) that can be assigned to the (2 1 1) and (2 2 2) planes of HAp.[8,32,33]

3.2. Cell attachment and proliferation of MC3T3-E1 cells

MC3T3-E1 cells were cultured on the PGA and PGA/HAp monoliths and the potential of these monoliths as a cell scaffold in bone tissue engineering was evaluated. For this experiment, the PGA/HAp monolith was used that had been pretreated with 1 M CaCl$_2$ in SBF for 7 d. A polystyrene culture plate was used as a control. At first, we tested whether the cells were able to attach on the PGA monolith using CLSFM. Cells were visualized with calcein AM and the monolith backbone had been labeled with Rhodamine B using the carboxylic acid groups of PGA. As can be seen in Figure S4, MC3T3-E1 cells attached and spread on the monolith surface.

The viability of cells cultured on the PGA and PGA/HAp monoliths was evaluated by the MTT assay (Figure 8). The cells proliferated continuously for 12 d on the PGA and PGA/HAp monoliths while the cell number reached a plateau after 9 d for a polystyrene plate. No significant difference in cell number was observed between the PGA and PGA/HAp monoliths. This continuous growth on the monoliths can be attributed to the submillimeter cavities which allow cell infiltration. Figure 9 shows CLSFM images of MC3T3-E1 cells on the monoliths stained with the LIVE/DEAD cell viability assay kit after 1 and 7 d of culture. As can be seen, live cells are covering the monolith backbone. Importantly, there were very few dead cells indicating that the monoliths were not cytotoxic which is in line with the MTT data. In addition, the cells

![Figure 5. EDX spectrum of the PGA/HAp monolith.](image-url)
Figure 6. FT-IR spectra of (a) PGA monolith and (b) PGA/HAp monolith.

Figure 7. XRD patterns of the (a) PGA monolith and (b) PGA/HAp monolith. Reference: JCPDS Card No. 74-0565.

Figure 8. Viability of MC3T3-E1 cells on the PGA, PGA/HAp monoliths, and a polystyrene plate ($n = 3$). *$p < 0.05$. 
seemed to be spreading more on the PGA/HAp monolith than the PGA monolith, which may be attributed to HAp deposited on the monolith surface. It is known that HAp improves cellular adhesion.\cite{34}

We also observed the localization of the cells within the PGA/HAp monolith after 7 d of culture. The three-dimensional CLSFM image revealed that the cells spread on the surface of the submillimeter cavities and did not migrate inside the microporous network (Figure S5 in the Supplementary material). To evaluate how far the cells migrated within the monolith after three weeks of culture, the PGA/HAp monolith was cut vertically to the surface and the cross-section was observed by CLSFM. As shown in Figure S6 in the Supplementary material, we observed cells at 600 μm below the surface. These data clearly showed that the cells can migrate inward through the sub-millimeter-sized cavities of the PGA/HAp monolith.

### 3.3. Loading and sustained release of BMP-2

BMP-2 is one of the important growth factors involved in the development and regeneration of bone and cartilage.\cite{35,36} This protein has an isoelectric point of 8.5 so that

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Figure 9. CLSFM images of MC3T3-E1 cells on the (a) PGA monolith and (b) PGA/HAp monolith after 1 d and 7 d. Green fluorescence: live cells and red fluorescence: dead cells.
it is positively charged at physiological pH 7.4. Since the PGA monolith possesses negative-charged carboxylate groups (pK\textsubscript{a} 4.8), it is expected that the monoliths can adsorb BMP-2 by electrostatic interaction.[37,38] Here we physically entrap BMP-2 to the PGA and PGA/HAp monoliths by simple electrostatic adsorption and evaluated their potential in controlled delivery of growth factors.

The PGA monolith and PGA/HAp monoliths were immersed in PBS containing BMP-2 (500 ng) and incubated at 4 °C. As shown in Figure S7, the concentration of BMP-2 in the supernatant decreased over 72 h. After 72 h of incubation, the concentration of BMP-2 was below detection limit of ELISA meaning that the BMP-2 had been completely absorbed by the monoliths. No significant difference in the absorbing capacity was observed between the PGA and PGA/HAp monoliths. To evaluate the internal localization of BMP-2 in the monolith, the BMP-2-loaded monolith was stained with fluorescamine. Fluorescamine is not fluorescent itself, but forms highly fluorescent products after reacting with primary amines. As shown in Figure S8 in the Supplementary material, fluorescence due to the presence of BMP-2 was observed both inner and outer side of the monolith.

We repeated the same experiment using twofold higher concentration of BMP-2 solution (PBS containing 1.0 μg of BMP-2). After 72 h of incubation, BMP-2 in the solution decreased to 0.1 ±0.04 μg meaning that the maximum loading amount per monolith was 900 ng. From this data, the amount of BMP-2 per volume of monolith was calculated to be 32 μg/mL (monolith volume: 28 μL). It has been reported that 0.2 mL of hyaluronan gels containing 4 μg of BMP-2, i.e. 20 μg/mL, could induce bone formation in rat.[39] Thus, the BMP-2 loading capacity of the monolith would be high enough for applications in bone tissue engineering.

The sustained release of BMP-2 from the monolith was confirmed in the presence of MC3T3-E1 cells. The cells were seeded onto the monoliths loaded with BMP-2 (500 ng) and cultured for 12 d replacing the culture medium every third day. As shown in Figure 10, both PGA and PGA/HAp monolith showed sustained release of BMP-2 without initial burst release. After 12 d of incubation, the amount of released BMP-2 was 0.33% (1.65 ng) for the PGA monolith and 0.32% (1.60 ng) for the PGA/HAp monolith, respectively. It has been reported that sustained release of BMP-2 promotes

Figure 10. BMP-2 release from the PGA monolith and PGA/HAp monolith (n = 3).
osteogenic differentiation and bone formation compared to fast release.\cite{37,38} Thus, this slow release of BMP-2 may be useful in bone healing which generally requires a long-term treatment for several months.\cite{40,41}

3.4. Alkaline phosphatase activity of MC3T3-E1 cells

The bioactivity of BMP-2 adsorbed within the PGA and PGA/HAp monoliths was confirmed using MC3T3-E1 cells by the ALP assay. MC3T3E-1 cells were cultured on the PGA and PGA/HAp monoliths loaded with BMP-2 (500 ng) for 12 d and the alkaline phosphatase (ALP) activity was evaluated, which is an early stage marker for osteogenic differentiation. As a control, cells were also cultured on a polystyrene plate for 12 d replacing the culture medium supplemented with BMP-2 (25 ng) every third day (total amount of BMP-2 added: 100 ng). As shown in Figure 11, in the absence of BMP-2, MC3T3-E1 cells showed a similar level of ALP activity for the PGA and PGA/HAp monoliths as well as the polystyrene plate. The addition of BMP-2 increased the ALP activity of the cells cultured on polystyrene. The PGA monolith loaded with BMP-2 showed comparable level of ALP activity to the polystyrene plate. Importantly, the cells showed 40% higher ALP activity when cultured on the PGA/HAp monolith compared to the PGA monolith and the polystyrene plate. Several reports have shown that the HAp surface enhanced the differentiation of preosteoblasts compared to plastic surfaces by promoting osteoblast adhesion, proliferation, differentiation, and extracellular matrix synthesis.\cite{11,42} Therefore, higher ALP activity of MC3T3-E1 cells on the PGA/HAp monolith may relate to the biological functions of HAp.

It is interesting to note that while the release of BMP-2 from the monolith was very slow and only 1.6 ng was released in the culture medium after 12 d, the ALP assay revealed that the BMP-2-loaded monolith showed similar or even higher bioactivity compared to the control (5 × 20 ng). Thus, although the detected BMP-2 in the culture medium was very low, it is possible that the adsorbed BMP-2 was released in the close vicinity of cells and/or directly interacted with cells to exert biological activity.

![Figure 11. ALP activity of MC3T3-E1 cells on the PGA monolith, PGA/HAp monolith, and polystyrene plate in the presence and absence of BMP-2 after 12 d of culture (n = 3).](image-url)
Furthermore, in addition to osteoblast differentiation and proliferation, angiogenesis is also essential in the process of bone repair.[43–45] It has been reported that porous hydrogel scaffolds with pores larger than 100–150 μm allow vascularized tissue formation.[46] As shown in Figure S3, the PGA/HAp monoliths possessed submillimeter-sized cavities (200–600 μm) which would be suited for vascularization. Therefore, the PGA/HAp monolith may also facilitate bone repair by allowing vascularization.

4. Conclusion

The PGA/HAp monolith was prepared by biomineralization of a PGA monolith in SBF. The HAp nucleation and crystal growth on the CaCl2-pretreated monolith was significantly faster than that of the untreated monolith. MC3T3-E1 cells efficiently attached and proliferated on both the PGA and PGA/HAp monolith without obvious cell toxicity. These monoliths efficiently absorbed BMP-2 by electrostatic interaction, which was slowly released into the medium in the presence of cells. Moreover, BMP-2 remained bioactive and MC3T3-E1 cells cultured on the BMP-2-loaded PGA/HAp monolith showed increased ALP activity compared to the PGA monolith. Therefore, the unique morphology and bioactivity of the PGA/HAp monoliths would be advantageous in bone tissue engineering.

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Supplemental data

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