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Ceramics International 39 (2013) 4991–4997

CERAMICS INTERNATIONAL

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Development and *in vitro* assays of porous calcium polyphosphate granules

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Received 11 September 2012; received in revised form 26 November 2012; accepted 28 November 2012
Available online 12 December 2012

Abstract

Porous calcium polyphosphate granules (CPPGs) for hard tissue regeneration were developed using amorphous calcium phosphate (CaP) glass particles. The glass particles were synthesised using a conventional glass melting technique, and the CPPGs were fabricated by a cement hardening process consisting of an acid–base reaction and a condensation reaction, which was developed in our previous studies. The pore sizes of the CPPGs were controlled using polymeric pore generators (porogens) of various sizes. The glass particles and CPPGs were analysed using X-ray diffractometry and Fourier transform infrared spectroscopy. The porosity of the CPPGs increased from 33.327% to 48.706% as the size of the porogens was decreased. To evaluate the cellular response to the developed porous CPPGs, cell proliferation and differentiation tests were performed, and an analysis of the relationship between the porosity and bioactivity was carried out. The results showed that both pore size and porosity influenced the osteogenic differentiation as well as the cell proliferation. This was due to the calcium ion release rate, which increased as the porosity increased and affected cellular behaviour. In conclusion, the porous CPPGs fabricated using amorphous CaP glass particles were shown to have potential as excellent bone graft materials.

Keywords: Amorphous calcium phosphate glass particle; Porous calcium polyphosphate granule; Cement reaction; Hard tissue regeneration

1. Introduction

Bone graft materials are widely used in orthopaedic and dental surgery to repair bone defects caused by fractures, removal of bone tumours and treat various congenital diseases [1]. Bone graft materials are generally divided into four types according to their properties (osteoconductivity, osteoinductivity and osteogenicity) and origin. Autologous grafts are known as clinical golden standard due to their excellent osteoinductivity and osteoconductivity, but they are limited due to donor-site morbidity, induced pain and limited availability of donor bone [2,3]. Allogenous and xenogenous grafts can be used as alternative materials; however, due to inconsistent quality and drawbacks related to pathology and immunogenicity, their limited potential for proper bone healing poses a major clinical issue [4]. Several synthetic biomaterials (alloplasts) have been developed to overcome these limitations and are widely used in clinical fields [5]. In the field of hard tissue regeneration, general trend is to use ceramic materials [6]. They are biocompatible while being osteoconductive, and materials such as calcium phosphate (CaP) closely resemble the composition of human bone [7,8]. They have also been applied as drug carriers as well as bone graft materials that showed antibacterial activity in our previous studies [9–11]. Those studies also introduced a new type of CaP bone graft material composed of amorphous CaP glass particles formed from basic materials and water by a cement setting reaction. The reaction consists of two steps, an acid–base reaction and a condensation reaction, that transform the original phosphate structure into a polyphosphate structure. These polyphosphate-structured materials have been applied as a granular-type materials; therefore, we named such materials as calcium polyphosphate granules (CPPGs). Previous studies have demonstrated the potential of CPPGs as bone graft materials, though consideration of their pore properties has been excluded.

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http://dx.doi.org/10.1016/j.ceramint.2012.11.096

There are many critical factors that play a role in hard tissue regeneration. In this study, we examined the presence of pores and their properties that influence the extent of bone formation [12]. It is known that porous matrices facilitate cell attachment, proliferation and mass transport [13]. In bone graft materials, representative properties of pores are size and interconnectivity. Pore size is related to neo-bone ingrowth, cell migration and proliferation, and matrix deposition in vacancies. Small pore sizes may impede cell adhesion and bone ingrowth. In fact, several studies have shown that the minimum required pore size is approximately 100 μm due to cell size and migration. Moreover, the pore size required to enhance the growth rate of neo-bone and the formation of capillaries is considered to be over 300 μm, and pore sizes in the range of 300–500 μm promote the mass transport of nutrients and vascularisation [12,14]. Interconnected pores provide channels for blood vessel formation as well as cell distribution and migration, while disconnected pores could be obstacles for blood vessel invasion [15].

In this study, we hypothesised that porous CPPGs could improve cell attachment, proliferation and differentiation in an *in vitro* environment. Under this hypothesis, porous CPPGs were prepared with interconnected pores using polymeric pore generators (porogens). The fundamental properties of the prepared porous structures were analysed, followed by an evaluation of cell attachment, proliferation and differentiation on the CPPGs in relation to the pore properties. The *in vitro* studies were performed in a 3D culture system to mimic the *in vivo* and the clinical environment. To our knowledge, this study is the first to evaluate porous CPPGs fabricated using amorphous CaP glass particles in a 3D *in vitro* culture system. Based on the results described below, the porous CPPGs may be considered as a promising alternative to synthetic bone graft materials.

2. Materials and methods

2.1. Fabrication of porous calcium polyphosphate granules

Amorphous CaP glass particles were synthesised using the same method described in previous studies [11,16]. Briefly, a glass batch of the system $CaO–P_2O_5–MgO–ZnO–CaF_2$ was prepared with a Ca/P ratio of 0.6. The raw materials $CaCO_3$ (Samchun Pure Chemical, Korea), H_3PO_4, MgO, ZnO (Duksan Pure Chemical, Korea), and CaF_2 (Junsei Chemical, Japan) were used, and the molar ratio of CaO/CaF_2 was fixed to 9. The batch was dried overnight at 100 °C and melted at 1250 °C for 2 h in a Kanthal Super furnace. After the batch was melted, it was subsequently quenched onto a graphite plate at room temperature. The amorphous CaP glass was crushed using an alumina mortar and was attrition milled using zirconia balls. An X-ray diffractometer (XRD; Ultima IV, Rigaku, Japan) and Fourier transform infrared spectroscopy system (FT-IR; Avatar 360, Thermo Nicolet, USA) were used to analyse the structure and organic matrix composition of the amorphous CaP glass particles. Particle size distribution analysis was performed using a dynamic light scattering technique (DLS; Zetasizer Nano ZS90, Malvern, UK).

Paraffin beads were prepared by the water-in-oil (w/o) method and used as the porogens [17]. Briefly, melted Paraplast® (Leica Microsystems, Germany) was added to a 1% polyvinyl alcohol (PVA; Sigma-Aldrich, USA) solution under vigorous stirring on a hot plate. This suspension was then poured quickly into iced water for solidification and then filtered through sieve with openings measuring 100 μm. The paraffin beads left on the sieve were then washed with distilled water to remove the residual PVA and lyophilised at −50 °C, 7 mTorr for a day. The dried paraffiin beads were filtered through sieves with openings of 850, 600, 425, 300 and 212 μm to obtain various sizes of paraffin beads. The nominal sizes of the openings were established by international standards [18].

Calcium polyphosphate structures were formed using the cement hardening process described in previous studies [9–11]. Briefly, the amorphous CaP glass particles were mixed with 7.5 wt% Na_2CO_3 (Duksan Pure Chemical, Korea) and mixed with paraffin beads in a 1.5 M NaOH solution in a 30/10/9 (g/g/mL; particles/beads/liquid) ratio. Sodium ions in the NaOH solution and Na_2CO_3 provided structural stability in the aqueous solution and an appropriate setting time. In this cement system, Na_2CO_3 also had various effects on the resulting material properties due to the decomposition of Na_2CO_3 through intermediates such as HCO^{3-}, H_2CO_3, and CO_2 gas, as well as OH^- and Na^+. The cement paste was moulded into a cylindrical shape and hardened by acid–base and condensation reactions under humid conditions at 37 °C for a day. After hardening, the samples were immersed in hexane (Duksan Pure Chemical, Korea) to leach out the paraffin phase. The cylindrical cement block was then crushed using a mortar; then, granules measuring 425–850 μm were sieved and collected. The FT-IR spectra of the material were analysed to compare the granules before and after leaching out the paraffin phase. The porosity of the CPPGs was measured using a mercury porosimeter (Autopore IV 9500, Micromeritics, USA), and their morphology was observed using a scanning electron microscope (SEM; JEM-2100F, JEOL, Japan). As shown in Table 1, the CPPGs were divided into five groups according to the sizes of the porogens.

Table 1
Group codes according to the porogen size and porosity of each group. The porosity increased with decreasing porogen size.

Porogen size (d, μm)	No porogen	212–300	300–425	425–600	600–850
Group code	NP	P2	P3	P4	P6
Porosity	–	48.706	43.899	41.379	33.327

2.2. Calcium ion release test

To generate calcium ion release profiles, the CPPGs were placed in a closed polystyrene round-bottom tube containing distilled water (40.0 mg/mL) and an ionic strength adjustor. Before the measurement, a calcium ion selective electrode (calcium ISE; Thermo Scientific, USA) was calibrated using supplied calcium calibration standards. The released calcium ions from each group were monitored using the calcium ISE for 21 days, and the water was replenished every day when the experiment was performed. The calcium ion concentration was expressed in parts per million (ppm), and the release profiles were plotted as a cumulative rate.

2.3. 3D culturing of pre-osteoblast MC3T3-E1

3D cell culturing was performed according to the methods described in a previous study by using cell culture inserts (NUNC, Denmark) composed of a polycarbonate membrane with a 3 μm pore size [19]. Forty milligrams of the CPPGs was placed in each insert. MC3T3-E1 mouse pre-osteoblasts (ATCC, USA) cultured on a cell culture dish were suspended in medium at a concentration of 1.0×10^5 cells/mL. One hundred microlitres of cell suspension was added to each insert and gently mixed by pipetting. A 24-well culture plate was filled with the culture medium (1.5 mL/well, *i.e.*, enough to immerse the granules), and the well plate was stored at 37 °C and 5% CO_2.

The initial adherent cells were observed using the LIVE–DEAD Viability Kit (Invitrogen, USA). After 6 h of seeding on the granules, the cells on granules were washed twice using phosphate buffer saline (PBS; Gibco, USA), and a 2 μM calcein AM solution was added to them. Images of the cells were obtained from a LSM 700 inverted confocal microscope equipped with the ZEN software programme (Carl Zeiss, Germany).

2.4. Measurements of cell proliferation and differentiation

Cell proliferation was performed using the Quanti-iT™PicoGreen® dsDNA Reagent and Kits (Invitrogen, UK) according to the manufacturer's instruction. On experimental days, the granules were washed 3 times with PBS, and seeded MC3T3-E1 were lysed using a 0.1% Triton X-100 solution (Sigma, Switzerland) in a micro-tube. Cells were frozen at −80 °C and thawed at 4 °C using a centrifuge at 12,000 rpm for 10 min to collect the supernatants. Equal volumes of the supernatants and dsDNA reagents were then placed into a black 96-well plate. The well plate was incubated for 5 min in the dark, and a reading was carried out with a microplate reader (POLARstar OPTIMA Micro-Plate Reader, BMG LABTECH, Germany) in fluorescence mode using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The results were calibrated using a standard curve plotted using the λDNA standard provided with the kits.

Cell differentiation was evaluated by measuring alkaline phosphatase (ALP) activity following 2 weeks of cell seeding. An ALP activity test was performed using a Sensolyte® *p*NPP Alkaline Phosphatase Assay Kit (AnaSpec, USA), and the results were normalised using the total protein amounts that were obtained by a bicinchoninic acid (BCA) assay. Briefly, the cells on the granules were rinsed twice with PBS and lysed using Triton X-100 at 4 °C for 10 min. The lysates were centrifuged at 2500g for 10 min at 4 °C, and the supernatants were placed in the measurement wells with the same amount of *p*NPP working solution. The mixture was allowed to react for 1 h at room temperature, and the absorbance was measured at 405 nm using a spectrophotometric plate reader (Epoch, BioTek, USA).

2.5. Statistical analysis

All data were statistically analysed using PASW Statistics 18 (SPSS, USA) and one-way ANOVA; Tukey's post-hoc doc test was then used to determine significant differences ($p < 0.05$).

3. Results and discussion

3.1. Properties of amorphous calcium phosphate glass particles

The properties of amorphous CaP glass particles are shown in Fig. 1. As shown in Fig. 1(a), XRD results for the particles confirm that no crystalline phase was present in

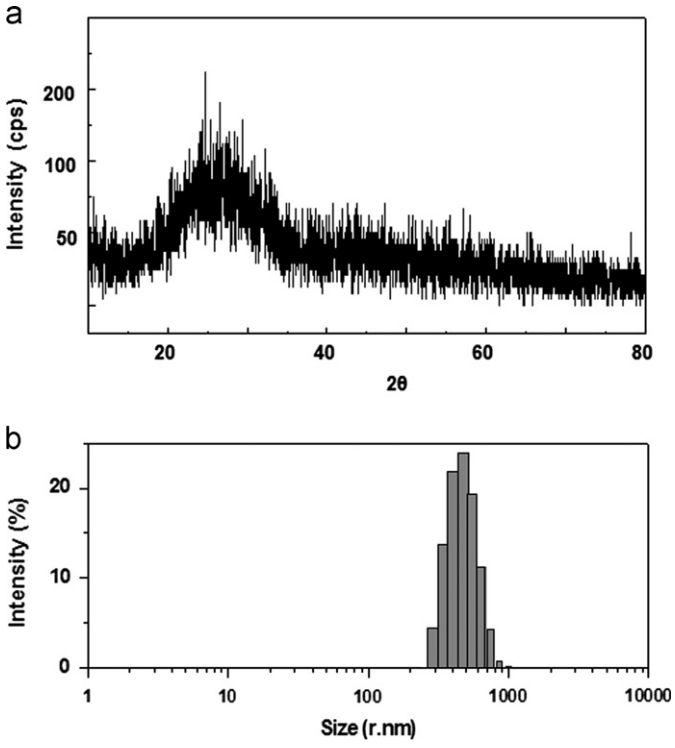

Fig. 1. (a) Typical amorphous CaPs XRD pattern and (b) size distribution of the amorphous CaP glass particles.

the samples; there was only a broad halo peak between 20° and 40°. Fig. 1(b) shows that the average radius of the particles was 458.0 ± 23.9 nm. Nano-sized particles are generally known to be more advantageous than micro-particles with respect to densification and solidification due to their specific surface area, and such enhanced densification and solidification is expected to improve the mechanical strength of the particles. The FT-IR spectrum of the particles is shown in Fig. 2, which shows vibration peaks of the phosphate structure (P–O and P–OH) between 1500 cm^{-1} and 700 cm^{-1}.

3.2. Characteristics of porous calcium polyphosphate granules

Porous CPPGs were fabricated using porogens of various sizes. Hexane was used to eliminate the polymeric porogens; hexane is a non-polar solvent that was naturally removed following the process by evaporation. Fig. 2 shows the FT-IR spectra of raw materials and each group of CPPGs. There were no peaks typical of hexane (–CH_3 in the region of 3000–2900 and 1500–1400 cm^{-1}) or paraffin (–CH_3 and –CH_2 in the region of 3000–2800 and 1500–1300 cm^{-1}) in the spectra of different groups of CPPGs. However, the amount of phosphate structures, shown in group NP, decreased slightly with the formation of pore structures, while the peaks, shown in organic phases, were eliminated in all of the groups. SEM images of the porous CPPGs are shown in Fig. 3. The vacancies by the porogens indented granules, except for those of group NP, and these structures led to changes in the porosity of each group (Table 1).

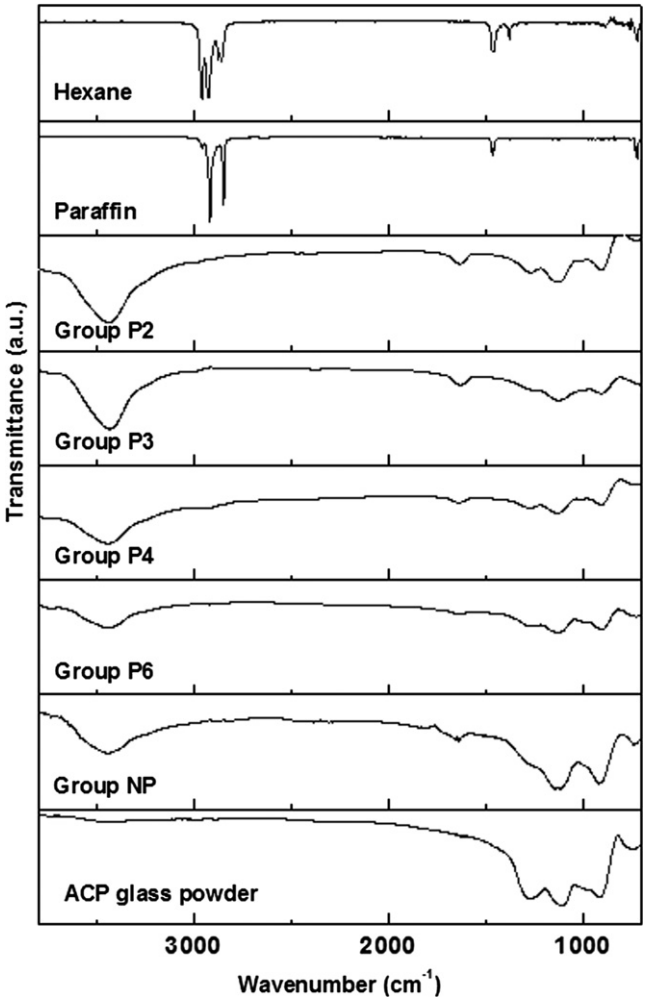

Fig. 2. FT-IR spectra of raw materials and fabricated CPPGs.

3.3. Calcium ion release profiles

The Ca^{2+} ion release rate is known to be related to bioactivity and biodegradability of biomaterials, and biodegradability is a critical requirement for ideal bone graft materials [4]. Biodegradability can induce and promote significant new bone formation by osteogenic cells [20]. Fig. 4 shows the Ca^{2+} ion release profiles of CPPGs produced in this study, which indicate that the release rate of porous groups was faster than that of group NP during the experiment; the release rate of group P2 in particular was overwhelmingly faster than that of group NP. However, clearly distinct release rates between the other porous groups (P2, P3, and P4) were not observed.

3.4. Cell adhesion and proliferation on porous calcium polyphosphate granules

As shown in Fig. 5(a), most of the cells were distributed on cavities formed by porogens. Such results indicate that pore structure is important for initial cell adhesion. The results of the PicoGreen assay are shown in Fig. 5(b). Columns in the figure indicate the amount of DNA measured, which can be interpreted as an index of cell proliferation. The group codes written in each column represent significant differences in cellular proliferation from the corresponding group. The results of first day of seeding on granules show that there was no linear increase in proliferation as the porosity was increased, but there were significant differences between some groups. On day 3, the amount of DNA sharply increased in group P2 and there were significant differences between group NP and all of the porous groups. On day 7, drastic increases were observed in all of the porous groups and there were significant differences between group NP and the porous groups, as observed on day 3. The significant differences between group NP and the porous groups seemed to be due to the grooves formed by the spherical porogens. This structure resulted in an increase in the surface area and porosity of the granules.

3.5. Osteogenic differentiation of MC3T3-E1 on porous calcium polyphosphate granules

To evaluate the level of osteogenic differentiation of MC3T3-E1 on porous CPPGs, ALP activity, which is

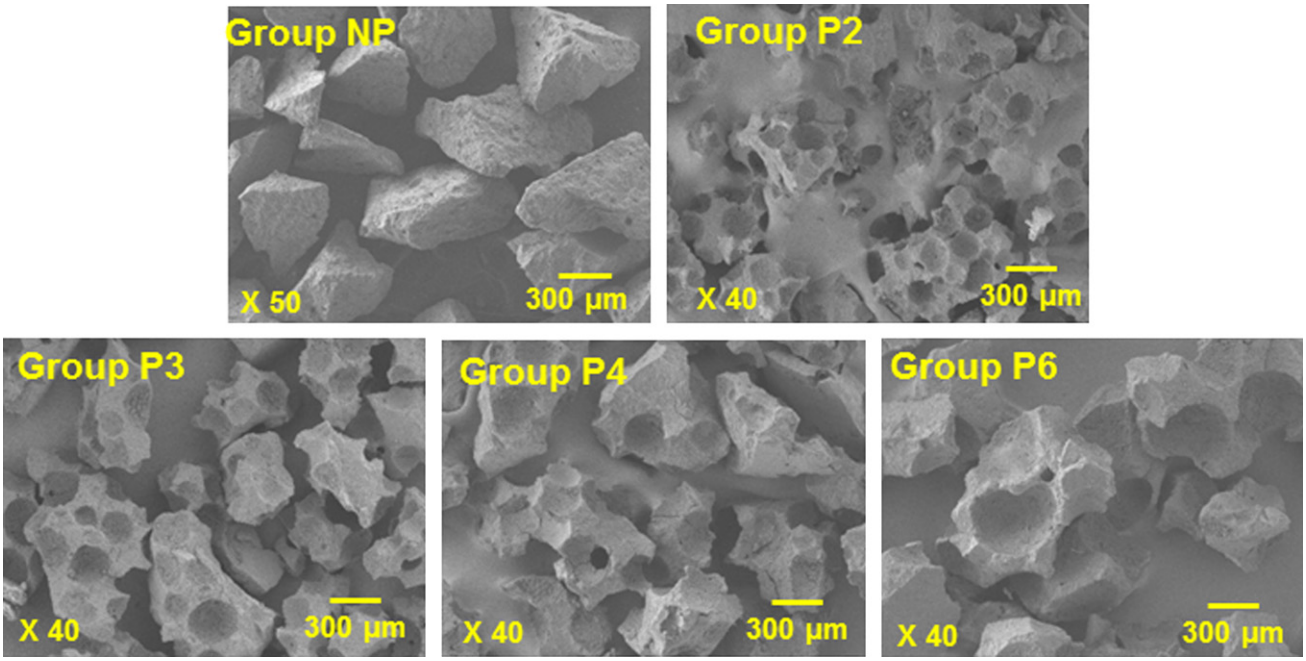

Fig. 3. SEM images of non-porous and porous CPPGs.

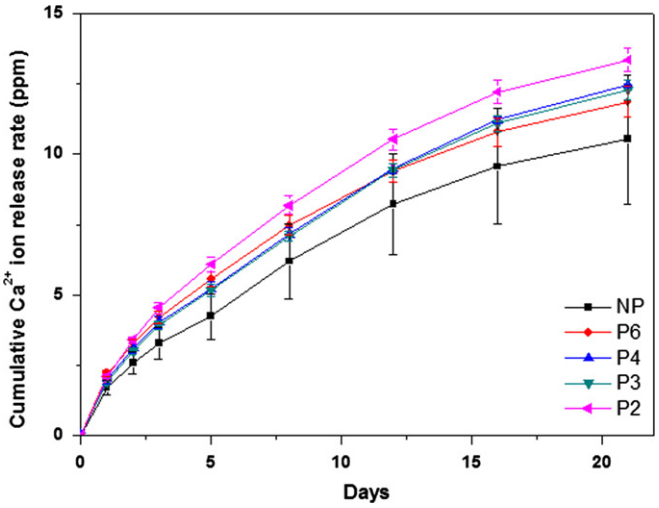

Fig. 4. Ca^{+2} ion release profiles of each group. Porous groups showed a faster release rate than the non-porous group on experimental days.

considered as an early marker of osteogenic differentiation related to osteocalcin for bone mineralisation [21], was measured following 1 and 2 weeks of cell seeding (Fig. 6). On day 7, the levels of ALP activity showed linearity with respect to the porosity of the granules, and there were significant differences in porosity. After 2 weeks of MC3T3-E1 seeding on the CPPGs, the ALP activity of all of groups recorded a threefold increase, and there was no significant difference between the groups. According to the results obtained on day 14, the CPPGs enhanced osteogenic differentiation, whether they were porous or not.

The result of cell proliferation and differentiation on day 7 showed that there were significant differences in the absolute values recorded as well as statistically significant differences. These results could be explained by previous studies [22–24], which concluded that Ca^{2+} ions play an essential role in promoting cellular migration and differentiation. According to the results of our Ca^{2+} ion release test, the release rate of group NP was slower than that of the porous groups, which could have affected the proliferation and osteogenic differentiation of MC3T3-E1 on the CPPGs on day 7.

4. Conclusions

In this study, amorphous CaP glass particles were developed using a conventional glass melting technique and porous CPPGs were fabricated using such particles. The pore size, which is known to be a critical factor in neo-bone regeneration, was controlled using paraffin beads of various sizes. Additionally, such granular-type bone graft materials show an interconnected pore structure and are expected to easily fill complex defect sites. In this study, it was shown that the porous structure of CPPGs was able to enhance cell proliferation, which is known as an initial stage of cell–materials interactions. An osteogenic differentiation study also revealed that the level of ALP activity increased with porosity, and there were significant differences in porosity. The results of this study indicate that the porous CPPGs can influence cellular responses, where the activity depends on pore properties and the apparent structure of the materials. We concluded that porous CPPGs are good candidates for use as alternative bone graft materials and will prove to be excellent biomaterials for clinical applications through future *in vivo* studies.

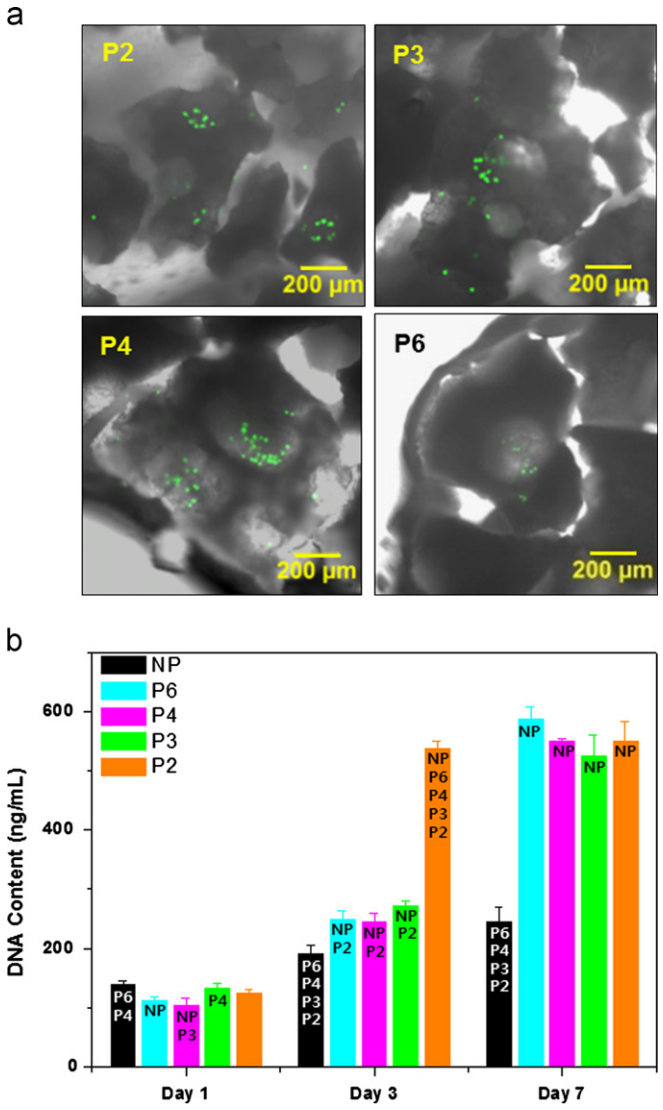

Fig. 5. (a) These images show that most of the cells adhere to the vacancies formed by porogens. (b) Results of PicoGreen assay show the extent of cell proliferation on the CPPGs. Group codes in the columns indicate that there are significant differences between groups ($p < 0.05$).

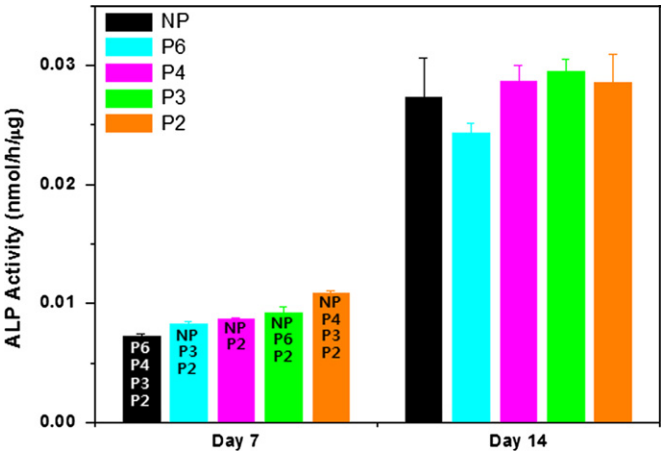

Fig. 6. ALP activity of MC3T3-E1 on the CPPGs. Group codes in the columns indicate that there are significant differences between groups ($p < 0.05$).

Acknowledgements

This study was supported by a grant of the Korea Healthcare Technology R&D Project, Ministry of Health, Welfare & Family Affairs, Republic of Korea (A101578). The authors would like to thank Dr. Jae-Sung Kwon M.D. for his helpful comments, and Ms. Song-Yi Yang for her excellent technical support. This study was carried out in part in the Yonsei-Carl Zeiss Advanced Imaging Center, Yonsei University College of Medicine.

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