



Indirect effects of calcium and phosphate ions releasing from Polycaprolactone – Biphasic Calcium Phosphate scaffolds on osteoblastic activities

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Abstract

Objective: To evaluate indirect effects of calcium and phosphate ions releasing from the Polycaprolactone (PCL) – Biphasic Calcium Phosphate (BCP) scaffolds fabricated by modified Melt Stretching and Multilayer Deposition (mMSMD) technique on proliferation and differentiation of osteoblasts.

Materials and Methods: The scaffolds were prepared as group A; PCL-20%BCP and group B; PCL-30%BCP (%wt). Amount of calcium and phosphate ions releasing from the scaffolds of both groups which immersed in culture medium (α -MEM) were assessed over 30 days. The effects of those ions on proliferation and differentiation of the osteoblasts cell lines (MC3T3-E1) were assessed using ELISA after culturing the cells in the medium with the immersed scaffolds over 21 days. The medium without scaffolds was used as the control group for all experiments.

Results: The release of calcium and phosphate ions from both groups remarkably increased on day 7 ($p < 0.05$) and then stable since day 14. No difference of their releasing profiles between the groups was detected ($p > 0.05$). The accumulative increase of those ions in both groups corresponded to their profiles of the cell proliferation and the levels of osteocalcin (OCN), but, the relationship was not found with the profiles of alkaline phosphatase (ALP). The ALP activity of group A increased with time and it was significantly higher than those of group B and the control group on day 21 ($p < 0.05$). In addition, the OCN levels of group A were higher than those of the other groups over the observation period.

Conclusion: PCL-BCP mMSMD scaffolds can sustain the releases of calcium and phosphate ions over the period of bone formation which are essential for supporting proliferation and differentiation of the osteoblasts. Those ions released from the PCL-20%BCP scaffolds would support the early and late phase of osteoblastic differentiation better than the PCL-30%BCP scaffolds, whereas, their effects on the cell proliferation are not different.

Keywords: Scaffold, Biphasic calcium phosphate, Hydroxyapatite, Tricalcium phosphate, calcium and phosphate ions

Introduction

For several decades, autogenous bone grafting for reconstructing large bone defects in maxillofacial region is still considered to be the gold standard, nevertheless, its requiring donor site operations sometimes increase patient morbidities. Therefore, several researchers play attention to develop synthetic biodegradable scaffolds used as bone substitutes instead of the autogenous bone aiming for reconstructing intra-oral bone defects. Ideally, the

scaffolds should act as temporary matrices for extracellular matrix deposition until the new bone is totally restored. The rate of degradation should be commensurate with bone regeneration whilst mechanical strength of the scaffolds should be maintained during this period. In addition, their structures should consist of appropriate pore size and interconnecting pore systems for transporting nutrition and bone cell in-growth throughout the scaffolds (Salgado, Coutinho, & Reis, 2004). For those purposes, our research team developed the novel



technique of Melt Stretching and Multilayer Deposition (MSMD) specifically for fabricating the biodegradable polymer-based scaffolds (Thuaksuban, Nuntanaranont, Pattanachot, Suttapreyasri, & Cheung, 2011; Thuaksuban et al., 2013). The MSMD scaffolds are designed to be an appropriate interconnecting pore system for enhancing osteogenesis. A microgroove pattern, typically found on their surfaces has proved to support attachment of osteoblasts (Thuaksuban et al., 2011). In addition, the mechanical properties of the scaffolds are suitable for withstanding forces occurring in real circumstances of the reconstruction in the oral and maxillofacial region (Thuaksuban et al., 2013). Recently, to make that fabricating process more practical, the steps of MSMD were simplified and so was renamed “modified MSMD (mMSMD)”. A three-dimensional (3-D) scaffold can be fabricated only by compressing a single filament into a glass mold and immersing in warm water. The mMSMD technique is easier to process which allowing any surgeons to instantly build up the 3-D scaffolds on chair side of the surgical operations. Therefore, time spending for the processing is remarkably reduced. In addition, fabricating the scaffold within the glass mold is a close environment that can prevent contamination during the processing. The concept of melt-blending of two materials which are Poly ϵ -caprolactone (PCL) as a major component and Biphasic Calcium Phosphate (BCP) as a filler is used for fabricating the composite scaffold. PCL has been approved by the Food and Drug Administration (FDA) as a medical and drug delivery device, with extensive support both in vitro and in vivo studies (Engelberg & Kohn, 1991; Lei, Rai, Ho, & Teoh, 2007; Rai, Teoh, & Ho, 2005; Schantz et al., 2003; Tay et al., 2007). It is degraded by a hydrolytic mechanism under physiological conditions and produces a less acidic environment when

compared to other polyesters. However, PCL normally takes more than 24 months for complete degrading, which is not commensurate with the bone remodeling process (Lam, Hutmacher, Schantz, Woodruff, & Teoh, 2009). BCP is a combination of a stable phase of hydroxyapatite (HA) and a soluble phase of beta-tricalcium phosphate (β -TCP) in different concentrations that offering controlled bioactivities and balanced biodegradation (Dorozhkin, 2010). Although the various composition ratios of these two materials have been studied extensively, the optimum ratio of BCP for clinical applications is still obscure. Most of the previous studies fabricated BCP using the ratios of HA higher than β -TCP which are mainly for improving mechanical stability of the materials, however, some studies demonstrated better results of those with the higher ratios of TCP (Arinzeh, Tran, Mcalary, & Daculsi, 2005; Ebrahimi, Pripatnanont, Suttapreyasri, & Monmaturapoj, 2014; Lomelino Rde et al., 2012; Nery, LeGeros, Lynch, & Lee, 1992). Arinzeh et al (Arinzeh et al., 2005) performed an in vivo study to determine the optimum ratio of HA and TCP for supporting human mesenchymal stem cells (hMSC) and inducing bone formation. Six types of ceramic including 100% HA, 100% TCP and BCP with the ratios of HA/TCP at 76/24, 63/37, 56/44, and 20/80 were seeded with hMSC and implanted subcutaneously into the backs of severely immunodeficient genetically disordered mice. The authors found that the BCP 20/80 had the better results when compared with the other proportions with the higher ratios of HA. Lomelino, et al (Lomelino Rde et al., 2012) evaluated the suitability of BCP granules (β -TCP/HA = 70:30) as potential carriers for cell-guided bone therapy. Calvarial bone defects (5 mm in diameter) of Wistar rats were filled with autogenous bone graft, the BCP granules combined with human bone cells and the BCP



granules alone. After 45 days, the new bone formation of the defects filled with the combination of the BCP granules and the cells were similar to those filled with the autogenous bone. Although the amounts of new bone formation in the group BCP granules alone were less than those of the other groups, but no significant difference was detected. Recently, in cooperation with The National Metal and Materials Technology Center of Thailand (MTEC), BCP particles were prepared as bone substitutes in different compositions of HA/ β -TCP including 30/70, 40/60 and 50/50 (Ebrahimi et al., 2014). Proliferation and differentiation of mouse osteoblast cells (MC3T3-E1) responding to those particles was assessed *in vitro* over 19 days. The results indicated that the cells which were seeded on the BCP 30/70 grew faster and expressed the highest alkaline phosphatase activity earlier than the other groups, whereas, the highest Osteocalcin activity was detected in the 50/50 group followed by 30/70 and 40/60 respectively. Therefore, it implies that the BCP containing higher ratios of TCP (30/70) would support proliferation and the early phase of differentiation of the osteoblast cells, whilst, higher HA ratios (50/50) would support the later phase. Those effects would be due to calcium and phosphate ions which can be released from the BCP particle. Ma et al (Ma et al., 2005) monitored dissolution behaviors of those ions released from plasma-sprayed HA coatings coating disks and assessed their effects on osteoblast precursor cell lines. The authors concluded that the cells responded differently to the different concentration of calcium and phosphate in the medium. In our opinion, BCP 30/70 is suitable to be used as the filler in the PCL-based scaffolds, not only for increasing bioactivities, but also for improving degradation property of the scaffolds. Regarding the mMSMD technique, BCP, which being stable within the temperature between 100–120°C,

is appropriate for the melt blending with PCL and the monofilaments of PCL–BCP blends can be fabricated homogeneously via an extruding machine (Thuaksuban et al., 2011). In this study, the bioactivities of the PCL–BCP scaffolds for supporting bone-forming cells were evaluated. Relations between the releasing ions from the scaffolds and growth and differentiation of the cells were observed and discussed.

Materials and Methods

Scaffold fabrication and study groups

The study groups were divided group A using the ratio of PCL: BCP = 80:20 (PCL–20%BCP) and group B = 70:30 (PCL–30%BCP). The PCL–BCP scaffolds were fabricated using the mMSMD technique as follows. In brief, PCL pellets (\bar{M}_n 80,000 PC, Sigma Aldrich, USA) and BCP particles (HA: β -TCP 30/70, particle sizes < 75 μm , MTEC, Pathumthani, Thailand) were used as raw materials. The two materials were mixed together in the ratios of PCL: BCP = 80:20 and 70:30 by weight and melted in a melting–extruding machine (Thuaksuban et al., 2011). The PCL–BCP monofilaments were made by extruding the PCL–BCP blend through the nozzle tip of the machine. After that, the filaments were stretched to decrease their diameter and then they were stocked for fabricating the scaffolds. To fabricate a 3-D scaffold (Figure 1), the single filament was cut into 50 cm in length and put into a 5 cc-glass syringe, and then, the plunger of the syringe was pushed until reaching the reference point of 3 mm above the bottom of the syringe. The tip of the syringe was sealed by polyvinyl siloxane (3M ESPE, USA), and then, it was immersed into warm double distilled water. By using this method, contacted surfaces of the filaments could be fused together and the 3-D scaffold (diameter: 11 mm, height: 3 mm) was built.



Morphologies of the scaffold specimens of all groups were demonstrated in figure 2. The specimens were sterilized using ethylene oxide gas 2 weeks prior to the experiments.

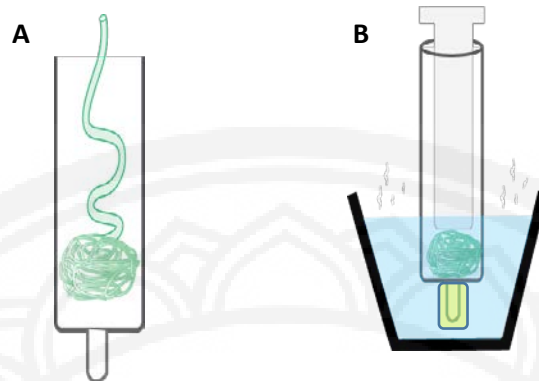


Figure 1 The fabrication process of the PCL–BCP scaffolds using the mMSMD technique; A: the PCL–BCP filament was put into a glass mold and compressed, B: the mold was immersed into warm water allowing the contact points of the filament to fuse together and form a 3–D scaffold.

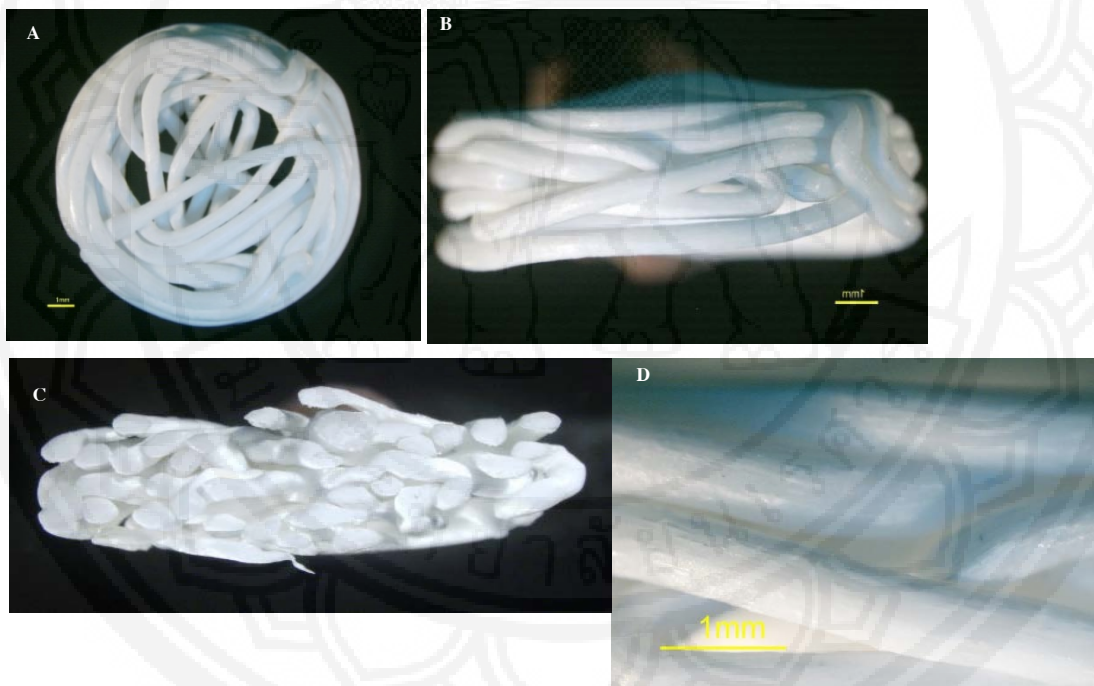


Figure 2 The stereomicroscope images demonstrated the morphologies of the PCL–BCP scaffold specimens; A: superior view, B: lateral view, C: cross–sectional view and D: the magnified picture focuses on the rough surface architecture of the scaffold.

Release of Calcium and Phosphate ions

The scaffolds of each group were left in the proliferation culture medium [Alpha–Minimum Essential Medium (α -MEM; Gibco, Invitrogen,

USA) containing 10% fetal bovine serum (FBS) (Gibco, Invitrogen, USA), 10000 units/ml penicillin/streptomycin (Gibco, Invitrogen, USA), and 250 μ g/mL fungizone (Gibco, Invitrogen,



USA)] (n=25/group) for detecting release of calcium and phosphate ions over 30 days. In brief, the scaffolds were immersed in 2 mL of the medium per well in a tissue culture plate and the plate was incubated at a constant temperature of 37°C, whilst, the mediums without the scaffolds were used as a control group. On day 3, 7, 14, 21 and 30 thereafter, the scaffolds were moved into the next wells and the fresh medium was added. The solution of each previous well was collected for measuring calcium and phosphate ions using a Calcium and Phosphate Colorimetric Assay Kit (Biovision, USA) (n=5/group/time point/testing). To detect the calcium ion, 90 µL of the Chromogenic Reagent and 60 µL of the buffer solution were added into 50 µL of each sample solution and mixed gently in a 96-well plate. The plate was incubated away from light for 5 min at room temperature. The absorbance (OD) of the chromophore was measured using a microplate reader (Thermo Fisher Scientific, Germany) at 575 nm. To detect the phosphate ion, 200 µL of the sample solutions were placed in the 96-well plate. Thirty microliters of phosphate reagent were added into each well and mixed gently, and then, the plate was incubated at room temperature for 30 min. The absorbance of Malachite Green and Ammonium Molybdate which formed a chromogenic complex with phosphate ions was measured at 700 nm. The levels of OD were compared with a standard curve to calculate the concentrations of calcium and phosphate released from the scaffolds.

Biocompatibility in vitro

Cell culture

Osteoblast cell lines (MC3T3-E1, subclone 4, ATCC, USA) were grown in the proliferation medium. The cells were cultivated in 5% CO₂ at 37 °C until reaching confluence, and then, subculturing was conducted. The cells between passages 3–6 were used for the experiments.

Cytocompatibility tests

The indirect cytocompatibility experiments were performed in order to evaluate an influence of the releasing ions from the scaffolds on proliferation and differentiation of the osteoblast cells. On day 21, 14, 7 and 3 prior to the experiment, 1 x 10⁴ cells were seeded into each well of the 48-well culture plates and then 200 µL of the proliferation medium were added (n=5/group/time point/testing). The plate were left for 3 h in 5% CO₂ at 37°C to allow for the cell attachment. After that, the scaffolds of both groups were immersed into each well and secured as close to the bottom of the well as possible. Five wells without the scaffold were reserved as the control group. The plates were cultivated in 5% CO₂ at 37°C and the medium was changed every 3 days until the experiments.

Cell proliferation; On the day of the experiment, the cell proliferative reagent (WST-1; Roche, Germany) was used to measure an activity of mitochondrial dehydrogenases for reflecting the number of viable cells as per the following protocol. For each well, the scaffold and culture medium were removed and replaced with 200 µL of the fresh culture medium without FBS and 20 µL of WST-1 solution. The well plates were incubated for 4 h in 5% CO₂ at 37°C. After that, 100 µL of the solution of each well was transferred to a 96-well plate in duplicate and the absorbance of the formazan product of each well was measured at 440 nm using a micro-plate reader. The levels of OD were compared with a standard curve to infer the amounts of the cells.

Cell differentiation; On the day of experiment, after removing the medium and the scaffold, the cells on the bottom of each well were washed two times using PBS, and then, they were lysed by freezing and thawing for three cycles (1 cycle: at - 20 °C for 15 min and at room temperature for 15 min). After that,



200 μ L of 1% Triton X-100 (Sigma, USA) in PBS were added and the mixtures were transferred into micro-centrifuge tubes. All tubes were centrifuged at 2000 x g for 10 min, and then, the supernatant of each tube was collected and kept at -80°C as the cell lysis solution used for an analysis of total cellular protein content, alkaline phosphatase activity (ALP) and osteocalcin assay (OCN). The quantification of total protein in the solutions were performed according to the manufacturer's instructions (Bio-Rad protein assay, USA) based on the method of Bradford. The absorbance at 750 nm was read using the micro-plate reader. The ALP activities were measured according to instructions using the commercial kit of Alkaline Phosphatase, AMP Buffer (Human, Germany) according to the recommendation of the International Federation of Clinical Chemistry (IFCC). Levels of the activity were calculated per one milligram of the total cellular protein [(U/L)/mg protein]. Quantification of OCN was performed according to the manufacturer instructions using the commercial kit of osteocalcin enzyme-linked immunosorbent assay (Biomedical Technologies Inc., USA). The solutions were read at 450 nm absorbance using the microplate reader and their concentrations were calculated with the serial diluted standard solution. The OCN levels were demonstrated as ng/mg protein.

Statistical analysis

The data was analyzed using statistics analysis software (SPSS, version 14.0, USA). One-way Analysis of Variance (ANOVA) followed by Tukey HSD was applied to compare the differences of the amounts of ions and the parameters of cytocompatibility tests among the experiment groups and the control group. Dunnett's T3 was performed when equal variances were not assumed. The level of statistical significance was set at a $p < 0.05$.

Results

Release of Calcium and Phosphate ions

The concentrations of calcium and phosphate ions released from the scaffolds were demonstrated in figure 3. The results demonstrated that the scaffolds of both groups could release those ions over the observation periods when immersed in the culture medium. In addition, there was no significant difference between the experiment groups over the time points ($p > 0.05$). It was noted that the releasing concentrations on day 7 were significantly increased more than other days ($p < 0.05$) and then decreased thereafter. The cumulative data demonstrated that the accumulation of those ions continued increasing with time and no significant difference was found between the experiment groups ($p > 0.05$).

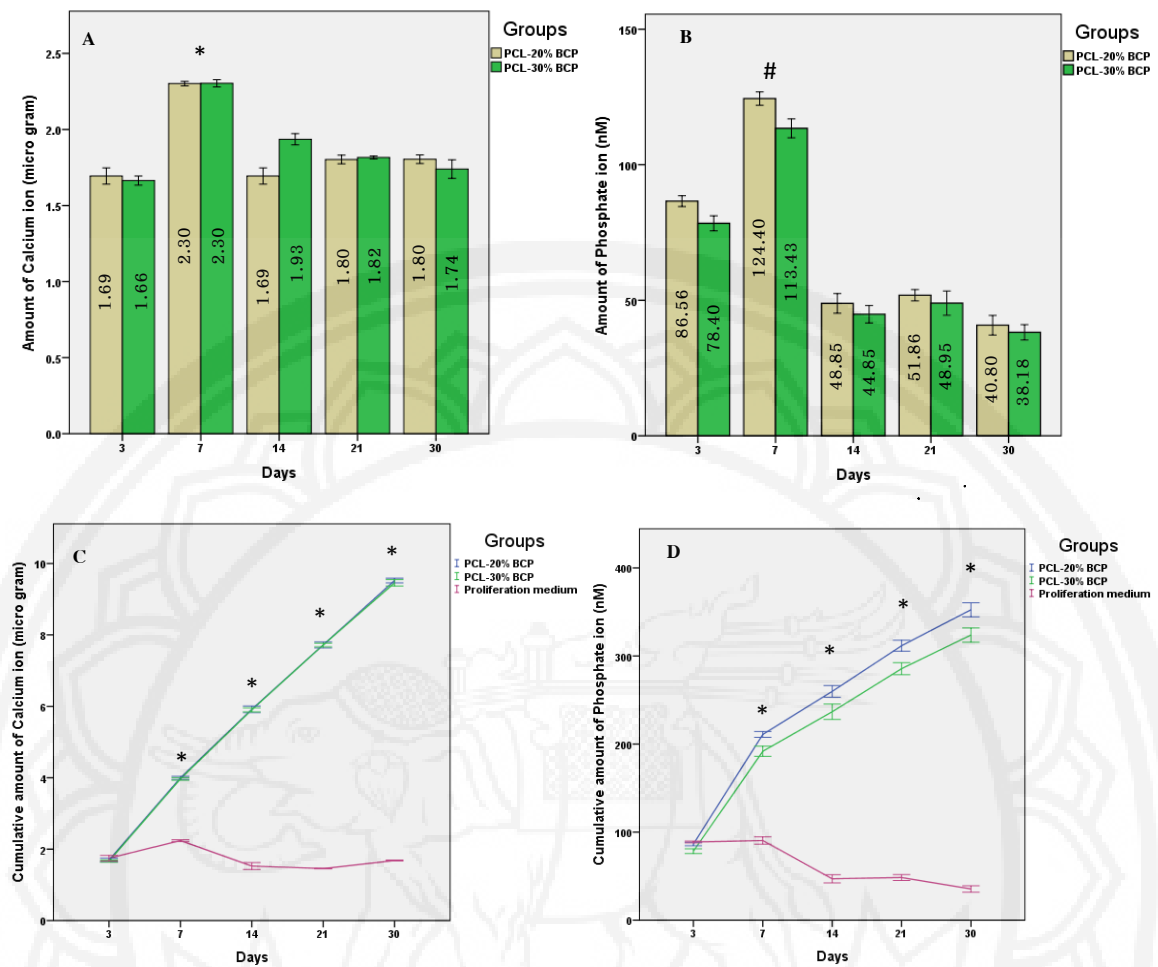


Figure 3 A and B; The graphs demonstrate profiles of calcium and phosphate ions released from the scaffolds over 30 days. The maximum release of both ions were detected on day 7, which was significantly greater than the other days (*, #; ANOVA, $p < 0.05$). The releasing decreased thereafter until reaching the lowest level on day 30. C and D; Accumulation of the released ions rapidly increased on day 7 and continued increasing with time thereafter. Since day 7, the amounts of those ions of the experiment groups were significantly greater that those in the culture medium (*; ANOVA, $p < 0.05$). In addition, there was no significant difference of those ions between the experiments groups over the observation periods.

Cytocompatibility tests

Cell proliferation

The amounts of the viable cells at all observation time points were demonstrated in figure 4. It was found that the amounts of the cells of the experiment groups slightly decreased on day 7, and then continued increasing thereafter to reach the maximum

growth on day 21. For the control group, the amounts of the cells on day 7 and 14 were significantly greater than those of the experiment groups ($p < 0.05$). The maximum growth of the cells was detected on day 14, but, it decreased on day 21 (significantly less than the experiment groups; $p = 0.00$)

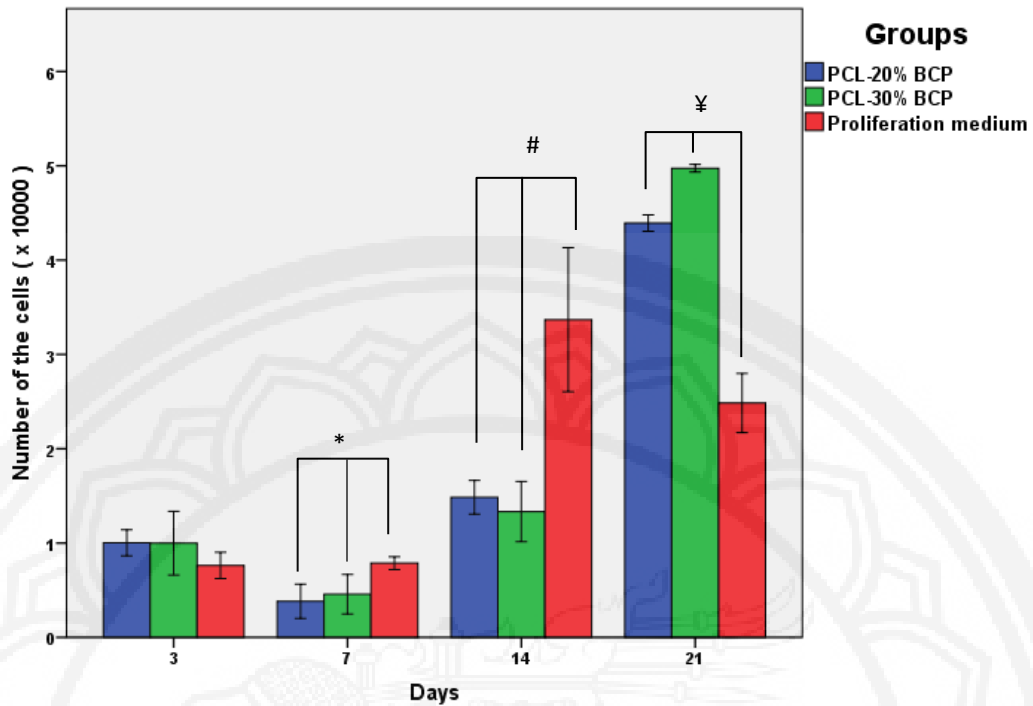


Figure 4 The bar graph shows that amounts of the cells in all groups slightly decreased on day 7, and then remarkably increased on day 14. On day 21, the amounts of the cells in proliferation medium + PCL-20% BCP scaffold and proliferation medium + PCL-30% BCP scaffold continued increased, whilst, those of the cells in proliferation medium alone decreased. (* and # = the viable cell number in proliferation medium was significantly greater than the other groups; ANOVA, $p = 0.00$ and 0.01 respectively. ¥ = the viable cell number in proliferation medium was significantly less than the other groups; ANOVA, $p = 0.00$).

Cell differentiation

The ALP activities of the cell-scaffold constructs were demonstrated in figure 5. The results demonstrated that the ALP levels of group A remarkably increased with time until reaching the maximum level on day 21 ($p > 0.05$). The maximum ALP level of group B was found only on day 3, but, it significantly decreased on day 7 and seemed to be stable on the following days ($p > 0.05$). The ALP levels of the control group seemed to be stable during

the observation periods. The OCN levels of those scaffolds were demonstrated in figure 6. It was found that the OCN levels of the experiment groups slightly decreased on day 14, but they remarkably increased thereafter. The maximum OCN level of the control group was detected on day 7, and then it decreased thereafter. It was noted that the OCN levels of the experiment groups were greater than those of the control group since day 14.

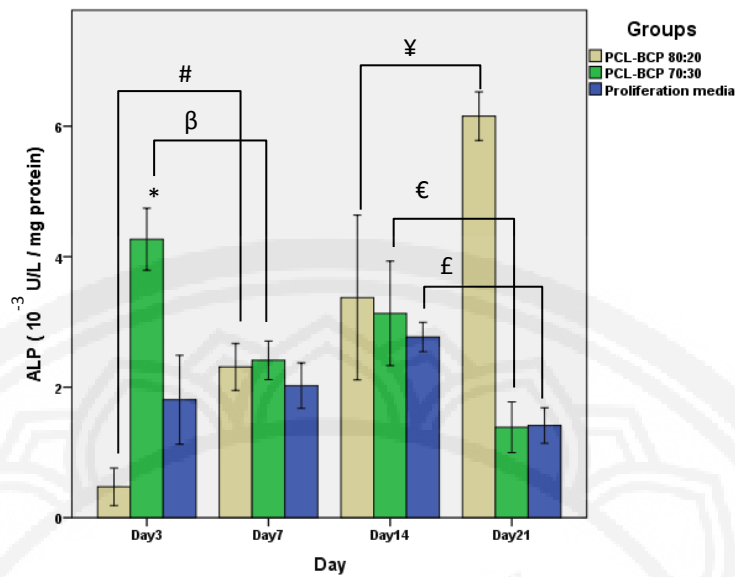


Figure 5 The bar graphs demonstrate ALP activities of the cell-scaffold constructs over 21 days. On day 3, the lowest levels of ALP were detected in proliferation medium + PCL-20% BCP scaffold, whilst, the maximum level was found in proliferation medium + PCL-30% BCP scaffold (significantly greater than the other groups, *; $p = 0.00$). On day 7, ALP activity of proliferation medium + 20% BCP scaffold significantly increased (#; $p = 0.029$), whilst, that of proliferation medium + PCL-30% BCP scaffold significantly decreased (β ; $p = 0.001$). On day 14, the levels of all groups slightly increased. On day 21, the ALP activity of proliferation medium + PCL-20% BCP scaffold significantly increase greater than those of the other groups (¥ ; $p = 0.00$), whilst, the ALP activity of proliferation medium + PCL-30% BCP scaffold and the cells in proliferation medium alone significantly decreased (€ and £ ; $p = 0.003$).

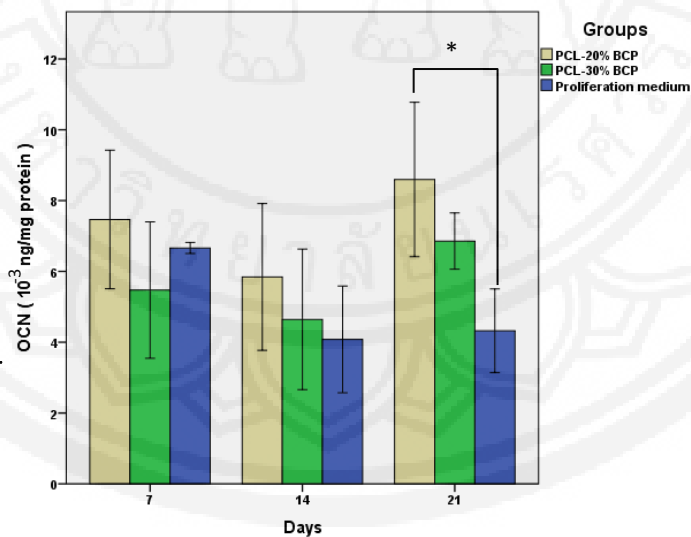


Figure 6 The bar graphs demonstrate the OCN levels of the cell-scaffolds constructs over 21 days. On day 7, the maximum level of OCN was detected in proliferation medium + PCL-20% BCP scaffold followed by the cells in proliferation medium alone and proliferation medium + PCL-30% BCP scaffold. On day 14, the levels of all groups slightly decreased. On day 21, those levels of proliferation medium + PCL-20% BCP scaffold and proliferation medium + PCL-30% BCP scaffold remarkably increase (* = significantly greater than the cells in proliferation medium, $p = 0.006$), whilst, the OCN level of the cells in proliferation medium alone was stable.



Discussion

This study revealed the indirect effects of the calcium and phosphate ions released from the PCL-BCP mMSMD scaffolds on the growth and differentiation of the osteoblasts. Similar to the MSMD technique, the maximum amounts of the BCP filler are not exceed 30 % due to an increase of fracture of the filaments (Thuaksuban et al., 2011). Therefore, the ratios of PCL-BCP at 70:30 and 80:20 were used for the experiments to evaluate the activities of the BCP filler. The result showed that the scaffolds of both ratios could sustain release of calcium and phosphate ions throughout 30 days. Although the maximum releasing of the ions was detected on day 7 and the rates of releasing decreased thereafter, the amount of those ions still cumulatively increased with time. Moreover, the cumulative concentrations of the experiment groups were significantly greater than those of the standard culture medium since day 7. Interestingly, there were no significant differences of the amounts of those ions and the profiles of their releasing between the experiment groups over the observation periods. Therefore, it implies that the ratio of 70:30 did not show any evident of more advantage in term of releasing more calcium and phosphate ions when compared with the ratio of 80:20. Regarding the result of the cell proliferation, the concentration of the calcium and phosphate ions seemed to correlate with the profiles of the cell growth. The burst releasing ions on day 7 seemed to produce an inappropriate environment for the growth of the cells, but decrease of the releasing rate thereafter would promote the cell proliferation until the day 21. Regarding the results of the cell differentiation, the ALP levels of the ratio of 80:20 continued increasing with time until reaching the maximum level on day 21, while, this profile was not found in

the groups of the ratio 70:30 and in the proliferation medium alone. The OCN levels of the experiment groups demonstrated the correlation of the ion concentrations and the changing profiles of OCN expression. It was found that the OCN levels of the ratio of 80:20 were higher than those of the ratio 70:30 over all time points. Therefore, it can be concluded that the ions released from the PCL-20%BCP scaffolds would be more suitable for supporting both of the early and late differentiation of the osteoblasts when compared with the PCL-30%BCP scaffolds, whilst, the effects of the two ratios on the cell proliferation were not different. Some previous studies demonstrated the effect of calcium and phosphate ions on behaviors of bone cells (Beck, Moran, & Knecht, 2003; Bingham & Raisz, 1974; Godwin & Soltoff, 1997; Maeno et al., 2005). For the effect of calcium ion, Maeno, et al (Maeno et al., 2005) investigated the effect of various concentrations of calcium ion on functions of cultured mouse primary osteoblasts. The result showed that the low concentration of calcium ion of 2-4 mM and 6-8 mM providing suitable conditions for proliferation and differentiation of the cells respectively. Godwin and Soltoff (Godwin & Soltoff, 1997) reported a relationship between extracellular calcium concentration and chemotaxis of MC3T3-E1 osteoblast cell line. They found that the rate of chemotaxis of the cells correlated with an increase of the calcium concentration within the range of 1.8 to 5 mM. For the effect of phosphate ion, Beck, et al (Beck et al., 2003) reported that inorganic phosphate is a signaling molecule for altering gene expression of the osteoblast cells during their differentiation. By using microarray analysis of phosphate-treated MC3T3-E1 osteoblast cell, they identified that some multiple genes such as Nrf2 which associating with the osteoblast differentiation were upregulated by an increase in the concentration



of phosphate ion. Bingham and Raisz (Bingham & Raisz, 1974) examined the effect of increasing phosphate ion in the range of 1.5 to 4.5 mM on bone growth and mineralization by using fetal rat long bones in organ cultures. They also found that increasing amounts of phosphate ion resulted in increased collagen content and calcification of the cultures. Regarding the results of our study, it can imply that the accumulative concentrations of calcium ion in the medium since day 14 are in the ranges of suitable environment for supporting growth of the osteoblast (Godwin & Soltoff, 1997; Maeno et al., 2005), whilst, those of the phosphate ion seem to be less than the optimum levels. Regarding economical and practical aspect in terms of saving the filler material and easier fabricating process, the ratio of PCL: BCP at 80: 20 is considered to be more suitable and it will be used for our future experiments.

Conclusion

The technique of mMSMD is very practical to fabricate PCL-BCP 3-D scaffolds. This study proves that the PCL-BCP mMSMD scaffolds have the osteoinductive property due to their sustainable release of the essential calcium and phosphate ions for supporting proliferation and differentiation of the osteoblasts over the period of normal bone formation. The concentration of those ions released from the PCL-20%BCP scaffolds would be better for supporting the entire phases of the osteoblastic differentiation when compared with the PCL-30%BCP scaffolds.

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