รายงานวิจัยฉบับสมบูรณ์

โครงร่างชนิดโพลีคาร์โปรแลคโตน-แคลเซี่ยมฟอสเฟตที่ผลิตด้วยเทคนิค เมลท์สเตรทชิงและมัลติเลย์เดพโพซิชัน เพื่อใช้ในงานวิศวกรรมเนื้อเยื่อกระดูก

The Polycaprolactone-Calcium Phosphate scaffolds fabricated by Melt Stretching and Multilayer Deposition technique for bone tissue engineering

คณะนักวิจัย

ผศ. ดร. ทพ. ณัฐวุฒิ เทือกสุบรรณ	หัวหน้าโครงการ
รศ.ทพญ. ปริศนา ปริพัฒนานนท์	ผู้ร่วมโครงการวิจัย
รศ.ทพ.นพ. ธงชัย นันทนรานนท์	ผู้ร่วมโครงการวิจัย

โครงการวิจัยนี้ได้รับทุนสนับสนุนจาก...งบประมาณแผ่นดิน มหาวิทยาลัยสงขลานครินทร์

ประจำปีงบประมาณ...2558.....รหัสโครงการ... DEN580250b.....

บทคัดย่อ

วัตถุประสงค์ เพื่อประเมินผลของแคลเซียมและฟอสเฟตไอออน ที่ถูกปลดปล่อยออกมาจากโครงร่างที่เป็นส่วนผสมของโพลีคาร์โปรแลคโตน และ ไบเฟสิคแคลเซียมฟอสเฟต ที่ผลิตด้วยวิธีเมลท์สเตรทชิง และ มัลติแลย์เดฟโพซิชัน แบบปรับปรุงใหม่ และผลของลักษณะของโครงร่างต่อการเจริญเติบโต และ การเปลี่ยนสภาพของเซลล์สร้างกระดูก

วัสดุและอุปกรณ์ ทำการเตรียมโครงร่างดังกล่าวโดยแบ่งออกเป็น 5 กลุ่มทดลองได้แก่ กลุ่ม A เป็นอัตราส่วนโดยน้ำหนักของโพลีคาร์โปรแลคโตน ต่อ ไบเฟสิคแคลเซียมฟอสเฟต ที่ 80 ต่อ 20 กลุ่ม B ใช้อัตราส่วน 70 ต่อ 30 กลุ่ม C ใช้โครงร่างที่มีส่วนผสมเป็นอัตราส่วนโดยน้ำหนักของโพลีคาร์โปรแลคโตน ต่อ ไตรแคลเซียมฟอสเฟต ที่ 80 ต่อ 20 กลุ่ม D ใช้อัตราส่วน 70 ต่อ 30 และกลุ่ม E ใช้โครงร่างที่มีส่วนผสมเป็นโพลีคาร์โปรแลคโตนเพียงอย่างเดียว ทำการวัดปริมาณของแคลเซียมและฟอสเฟตไอออน ที่ถูกปลดปล่อยออกมาจากโครงร่างในกลุ่ม A ถึง D โดยวัดความเข้มข้นของไอออนดังกล่าวในอาหารเลี้ยงเซลล์มาตรฐานชนิด แอลฟา-เอ็มอีเอ็ม ที่แช่โครงร่างในกลุ่มดังกล่าวไว้ภายในเวลา 30 วัน จากนั้นศึกษาผลต่อเซลล์สร้างกระดูกโดยอ้อมโดยทำการประเมินการเจริญเติบโต และการเปลี่ยนสภาพของเซลล์สร้างกระดูกชนิด เอ็มซี 3 ที 3-อี1 ด้วยวิธีอีไลซ่า และการย้อมสีผลึกแคลเซียมที่สร้างจากเซลล์ด้วยอะลิซารินเรดเมื่อทำการเพาะเลี้ยงเซลล์ดังกล่าวด้วยอาหารเลี้ยงเซลล์ที่แช่โครงร่างกลุ่มดังกล่าวไว้ภายในเวลา 21 วัน โดยเปรียบเทียบผลกับกลุ่มควบคุม คืออาหารเลี้ยงเซลล์ที่ไม่ได้แช่โครงร่าง ส่วนการศึกษาผลโดยตรงจะทำการเพาะเลี้ยงเซลล์ดังกล่าวบนผิวของโครงร่างในกลุ่ม A B และ E ภายในเวลา 30 วัน โดยแบ่งเป็นเลี้ยงในอาหารเลี้ยงเซลล์มาตรฐาน และในอาหารเลี้ยงเซลล์ที่เพิ่มสารกระตุ้นการเปลี่ยนสภาพเซลล์ เพื่อเปรียบเทียบการยึดเกาะ การเจริญเติบโต และการเปลี่ยนสภาพของเซลล์บนโครงร่างดังกล่าวด้วยวิธีการอีไลซ่า และ การส่องดูด้วยกล้องจุลทรรศน์อิเลกตรอนชนิดส่องกราด โดยใช้กลุ่ม E เป็นกลุ่มควบคุม

ผลการศึกษา โครงร่างในกลุ่ม A ถึง D สามารถปลดปล่อยแคลเซียม และ ฟอสเฟตไอออนออกมาได้ตลอดเวลา 30 วันที่ทำการทดสอบ โดยอัตราการปลดปล่อยแคลเซียมไอออนจะสูงสุดในวันที่ 14-21 และ ฟอสเฟตไอออนในวันที่ 3-7 หลังจากนั้นอัตราการปลดปล่อยจะลดลง เมื่อดูระดับความเข้มข้นสะสมของไอออนพบว่า กลุ่ม A และ C สามารถปลดปล่อยฟอสเฟตไอออนสะสมได้มากกว่ากลุ่ม B และ D อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ในทุกช่วงเวลา ส่วนการปลดปล่อยแคลเซียมไอออนสะสมจะไม่แตกต่างกันในทุกกลุ่มทดลอง สำหรับการศึกษาผลต่อเซลล์โดยอ้อมพบว่า เมื่อดูการเพิ่มจำนวนของเซลล์และการเปลี่ยนสภาพของเซลล์โดยวัดจากจากปริมาณอัลคาไลน์ฟอสฟาเตส และ ออสทีโอแคลซินของทุกกลุ่มทดลองพบว่ามีความสัมพันธ์กับปริมาณไอออนสะสมที่ปลดปล่อยออกมาของโครงร่าง โดยการเจริญเติบโตของเซลล์ในทุกกลุ่มทดลองซึ่งลดลงในวันที่ 7 จะสัมพันธ์กับการปลดปล่อยฟอสเฟตไอออนที่สูงสุดในช่วงเวลาดังกล่าว และตั้งแต่วันที่ 14 การเจริญเติบโตของเซลล์ที่เพิ่มขึ้นอย่างต่อเนื่องจนถึงระดับสูงสุดในวันที่ 21 จะสัมพันธ์กับการปลดปล่อยแคลเซียมไอออนที่เพิ่มขึ้นและฟอสเฟตไอออนที่ลดลง ส่วนปริมาณอัลคาไลน์ฟอสฟาเตสในกลุ่ม A และ D จะเพิ่มขึ้นตามเวลา โดยตั้งแต่วันที่ 14 ค่าดังกล่าวของกลุ่ม A จะมากกว่ากลุ่มอื่น ๆ และมากกว่าอย่างมีนัยสำคัญทางสถิติในวันที่ 21 ($p < 0.05$) ส่วนค่าใน B และ C ค่อนข้าง

คงที่ตลอดระยะเวลา 21 วัน เมื่อประเมินระดับออสทีโอแคลซินพบว่า ค่าในกลุ่ม A และ B จะค่อนข้างคงที่ใน 14 วันแรก และจะเพิ่มขึ้นอย่างชัดเจนในวันที่ 21 ส่วนค่าในกลุ่ม C และ D จะค่อนข้างคงที่ตลอดระยะเวลาที่ศึกษา โดยค่าในกลุ่ม C จะมากกว่ากลุ่มอื่น ๆใน 14 วันแรก แต่ค่าในกลุ่ม A จะมากกว่ากลุ่มอื่น ๆ ในวันที่ 21 สำหรับการวัดปริมาณผลึกแคลเซียมที่สร้างจากเซลล์พบว่า ทุกกลุ่มทดลองจะค่อนข้างคงที่ในช่วง 14 วันแรก และเพิ่มขึ้นอย่างชัดเจนในวันที่ 21 ซึ่งมากกว่าค่าในกลุ่มควบคุมที่เลี้ยงในอาหารเลี้ยงเซลล์มาตรฐานอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) แต่ยังคงน้อยกว่าเซลล์ที่ถูกเลี้ยงในอาหารเลี้ยงเซลล์ที่เพิ่มสารกระตุ้นการเปลี่ยนสภาพเซลล์ในทุกช่วงเวลา สำหรับการศึกษาผลโดยตรงพบว่า เมื่อดูด้วยกล้องจุลทรรศน์อิเลกตรอนชนิดส่องกราด เซลล์สร้างกระดูกที่ทำการเพาะเลี้ยงบนพื้นผิวของโครงร่างในกลุ่ม A B และ E มีการยึดเกาะ และเจริญเติบโตได้เป็นอย่างดี โดยในอาหารเลี้ยงเซลล์มาตรฐาน การเจริญของเซลล์ในกลุ่ม A จะเพิ่มตามเวลาจนมีค่าสูงสุดในวันที่ 30 ส่วนในกลุ่ม B จะเพิ่มจนมีค่าสูงสุดในวันที่ 14 หลังจากนั้นจะลดลง นอกจากนั้นจะพบว่า ค่าดังกล่าวในกลุ่ม A และ B จะมากกว่ากลุ่ม E ในทุกช่วงเวลา ส่วนในอาหารเลี้ยงเซลล์ที่เพิ่มสารกระตุ้นการเปลี่ยนสภาพเซลล์พบว่า กลุ่ม A และ B มีอัตราการเพิ่มจำนวนของเซลล์ไม่ค่อยชัดเจนแต่ยังคงมากกว่ากลุ่ม E ยกเว้นเฉพาะในวันที่ 21 เมื่อดูระดับของอัลคาไลน์ฟอสฟาเตสของเซลล์เมื่อเลี้ยงในอาหารเซลล์เลี้ยงมาตรฐาน พบว่า ค่าในกลุ่ม A และ B จะสูงใน 7 วันแรก และจะลดลงในวันที่ 14 ถึง 21 หลังจากนั้นจะเพิ่มขึ้นอย่างชัดเจนอีกครั้งในวันที่ 30 เมื่อดูค่าดังกล่าวในอาหารเลี้ยงเซลล์ที่เพิ่มสารกระตุ้นการเปลี่ยนสภาพเซลล์พบว่า ค่าในกลุ่ม A และ B จะเพิ่มขึ้นตามเวลาจนมีค่าสูงสุดในวันที่ 14 หลังจากนั้นจะค่อนข้างคงที่ เมื่อดูระดับของออสทีโอแคลซินของเซล์เมื่อเลี้ยงในอาหารเลี้ยงเซลล์มาตรฐาน พบว่า ค่าของกลุ่ม A และ B จะมีค่าค่อนข้างคงที่ใน 21 วันแรก และจะเพิ่มขึ้นอย่างชัดเจนในวันที่ 30 ในขณะที่ค่าในกลุ่ม E จะค่อนข้างคงที่ตลอด 30 วัน และเมื่อเลี้ยงเซลล์ในอาหารเลี้ยงเซลล์ที่เพิ่มสารกระตุ้นการเปลี่ยนสภาพเซลล์พบว่า ในกลุ่ม A และ B พบลักษณะการเพิ่มของระดับออสทีโอแคลซินคล้ายกับการเลี้ยงในอาหารเลี้ยงมาตรฐาน ส่วนในกลุ่ม E จะมีค่าเพิ่มขึ้นเล็กน้อยในวันที่ 30 เป็นที่น่าสนใจว่าค่าของตัวบ่งชี้การเปลี่ยนสภาพของเซลล์ทั้งสองชนิดนี้ของกลุ่ม A และ B จะมากกว่าค่าในกลุ่ม E ในเกือบทุกช่วงเวลาที่ศึกษา ไม่ว่าจะเลี้ยงเซลล์ในสภาวะมาตรฐาน หรือ สภาวะที่มีการกระตุ้นการเปลี่ยนสภาพเซลล์

สรุปผลการศึกษา โครงร่างที่เป็นส่วนผสมของโพลีคาร์โปรแลคโตน และ ไบเฟสิคแคลเซียมฟอสเฟต ที่ผลิตด้วยวิธีเมลท์สเตรทชิง และ มัลติแลย์เดพโพซิชัน แบบปรับปรุงใหม่ สามารถปลดปล่อย แคลเซียม และ ฟอสเฟต ไอออน ซึ่งมีส่วนสำคัญต่อการเจริญเติบโต และ การเปลี่ยนสภาพของเซลล์สร้างกระดูกได้ตลอดระยะเวลา 30 วันซึ่งเป็นระยะเวลาปกติของการสร้างกระดูกใหม่ โดยโครงร่างที่มีไบเฟสิคแคลเซียมฟอสเฟตผสมอยู่ร้อยละ 20 สามารถปลดปล่อยไอออนในปริมาณที่เหมาะสมต่อการส่งเสริมการเปลี่ยนสภาพของเซลล์สร้างกระดูกทั้งในช่วงต้น และ ช่วงปลาย ได้ดีกว่าโครงร่างที่ปริมาณไบเฟสิคแคลเซียมฟอสเฟตอยู่ร้อยละ 30 ส่วนผลต่อการเจริญเติบโตของเซลล์นั้นไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ นอกจากนั้นลักษณะรูปร่างของโครงร่างที่ผลิตด้วยวิธีการนี้ยังมีความเหมาะสมต่อการยึดเกาะ การเจริญเติบโต และการเปลี่ยนแปลงสภาพของเซลล์สร้างกระดูก โดยโครงร่างโพลีคาร์โปรแลคโตนที่มีไบเฟสิคแคลเซียมฟอสเฟตเป็นส่วนผสม จะมีความเหมาะสมต่อการการเจริญเติบโต และการเปลี่ยนสภาพของเซลล์มากกว่าโครงร่างที่ไม่มีส่วนผสมดังกล่าว

Abstract

Objectives: To evaluate 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 and the scaffold architectures on proliferation and differentiation of osteoblasts.

Materials and Methods: The scaffolds were prepared as group A; PCL-20%BCP (%wt), group B; PCL-30%BCP, group C; PCL-20% Tricalcium Phosphate (TCP), group D; PCL-30% TCP and group E; 100% PCL. Amounts of calcium and phosphate ions released from the scaffolds of group A-D, which immersed in the proliferation culture medium (PR, Alpha-MEM) were assessed over 30 days. For the indirect cytocompatibility test, the effects of those ions on proliferation and differentiation of osteoblasts cell lines (MC3T3-E1) were assessed by using ELISA and mineralized matrix staining (Alizarin Red S staining, AR) after culturing the cells in the medium with the immersed scaffolds over 21 days. The PR medium and osteogenic culture medium (OS, Alpha-MEM + inductive reagents) alone were used as the control groups. For direct cytocompatibility test, the cell-scaffold constructs of group A, B and E were made by seeding the cells on the scaffold surfaces and cultured in PR and OS mediums for 30 days, whilst, the scaffolds of group E were used as the control group. Proliferation and differentiation of those cells cultured in both mediums were assessed using ELISA and Scanning Electron Microscope (SEM).

Results: Scaffold in group A-D could sustain release of calcium and phosphate ions over 30 days. The maximum releasing rates of calcium ion were detected on day 14 – 21, whilst, those of phosphate ion were detected on day 3 - 7. For cumulative release of the ions, the scaffolds of group A and C released phosphate ion significantly higher than group B and D over the time points ($p<0.05$), whilst, the profiles of calcium ion among the groups were not significantly different. For the indirect cytocompatibility test of group A – D, proliferation and differentiation markers of the cells detected by measuring the levels of Alkaline Phosphatase (ALP) and Osteocalcin (OCN) seemed to relate to the profiles of the cumulative concentrations of the ions. The proliferation of the cells in all groups decreased on day 7 relating to the maximum releasing of phosphate ion, whilst, an increase of proliferation of the cells thereafter would relate to the releasing profiles of calcium ion. The ALP activities of group A and D increased with time and the level of group A was significantly higher than the other groups on day 21($p<0.05$), whilst, those of group B and C were stable over 21 days. The levels of OCN in group A and B were stable during the first 14 days, and then, remarkably increased on day 21, whereas, those of group C and D were stable over the observation period. It was found that the levels of OCN in group C were greater than those of other groups on the first 14 days, whereas, the levels in group A was higher than the other groups on day 21. The levels of the solubilized AR staining of all groups were stable during the first 14 days, and then, remarkably increased on day 21. At that time, those levels were significantly greater than that of the PR medium ($p<0.05$), but, still less than that of the OS medium. For the direct cytocompatibility test, the SEM images demonstrated that the osteoblast cells could attach and grow well on the surfaces of the scaffolds of group A, B, and E. In the PR medium, the viable cell numbers in group A increased with time and reached the maximum level on day 30, whereas, those in group B reached the maximum level on day 14, and then, decreased thereafter. It was noted that the growth of the cells in group A and B were higher than that of group E over the time points. In the OS medium, the proliferation rates of group A and B were not stable, but still higher than those of group E, except only on day 21. The ALP activities in group A and B when

cultured in the PR medium were detected at the high levels on the first 7 days, then decrease on day 14 - 21 and became remarkably increasing on day 30. In the OS medium, the ALP activities of group A and B increased with time and reached the maximum levels on day 14, and then became stable thereafter. The OCN levels of group A and B were stable during the first 21 days, and then, they remarkably increased on day 30 when cultured in the PR medium, whereas, those of group E were stable over 30 days. In the OS medium, the profiles of OCN in all groups were similar to those in the PR medium, whereas, those of group E slightly increased on day 30. It was noted that those osteoblastic differentiation markers of group A and B were higher than those of group E in almost all time points over the observation period whether cultured in the PR or OS medium.

Conclusion: The PCL-BCP mMSMD scaffolds can sustain release of calcium and phosphate ions which are essential for supporting the functions of the osteoblasts over the period of bone formation. The PCL- 20% BCP scaffolds can release the ions in the proper concentrations for enhancing the entire phased of the osteoblastic differentiation better than the PCL-30%BCP mMSMD scaffolds did, whereas, the effects on the cell proliferation are not statistically different. In addition, the morphologies of the PCL-BCP mMSMD scaffolds are more suitable for supporting attachment, growth and differentiation of the osteoblast cells than those of the scaffolds without the BCP filler.

Keywords: Scaffold, Polycaprolactone, Biphasic calcium phosphate, Hydroxyapatite, Tricalcium phosphate, calcium ion, phosphate ion

Content

Introduction

For several decades, using autogenous bone grafting for reconstructing large bone defects in the maxillofacial region has been considered to be the gold standard, nevertheless, its requiring donor site operations that sometimes increases patient morbidities. Therefore, several oral surgeons require some synthetic biodegradable scaffolds used as bone substitutes instead of the autogenous bone for those purposes. Ideally, the scaffolds should act as temporary matrices for extracellular matrix deposition until new bone is totally restored. The rate of degradation of the scaffolds should be commensurate with the bone regeneration, whilst, their mechanical strength should be maintained during that period. In addition, their structures should consist of appropriate pore size and interconnecting pore systems for transporting nutrition and allowing bone cell in-growth throughout the scaffolds (1). Since 2011, our research team has developed the technique of Melt Stretching and Multilayer Deposition (MSMD) specifically for fabricating the biodegradable polymer-based scaffolds (2-4). The MSMD scaffolds are designed to have the appropriate interconnecting pore system for enhancing osteogenesis. The microgroove pattern, typically found on their surfaces has proved to support attachment of osteoblasts (2). 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(3). Recently, to make that fabricating process more practical, the steps of the MSMD technique are simplified and it is 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 the chair side of 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. For the materials, the concept of melt blending of two materials including Poly ε-caprolactone (PCL) as a major component and Biphasic Calcium Phosphate (BCP) as a filler, is used for fabricating the composite scaffold. Poly ε-caprolactone has been approved by Food and Drug Administration (FDA) as a medical and drug delivery device, with extensive support both in vitro and in vivo studies (5-10). 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 (11). Biphasic Calcium Phosphate is a combination of a stable phase of hydroxyapatite

(HA) and a soluble phase of beta-tricalcium phosphate (TCP) in different concentrations that offering controlled bioactivities and balanced biodegradation (12). Bioactivities, mechanical properties and degradation behaviors of BCP are controlled by varying the ratios of the HA and TCP. With the higher ratios the TCP, the higher degradation rates can be archived, on the other hand, with the more composition of HA, BCP has better mechanical strength. In addition, BCP can prolong release of calcium and phosphate ions as well as forming hydroxyl carbonate apatite layer which are essential for the process of new bone formation (13, 14). Present of HA on surfaces of scaffolds can also change their surface charges and promote absorption of proteins and other molecules from surrounding environment (15-17). In bone tissue, the proteins such as osteopontin, bone sialoprotein, and osteocalcin are able to recognize HA through highly acidic domains, resulting in promoting attachment, proliferation and differentiation of osteogenic cells which subsequently forming new bone (15, 18-21). Additionally, the releasing calcium ion is able to neutralize the adverse acidic by-products during degradation of the polyester-based scaffolds (15, 22-24). Although the various ratios of the BCP have been studied extensively, its optimum ratio for bone tissue engineering and clinical applications is still obscure. Most of the previous studies fabricated BCP using the ratios of HA higher than TCP which is mainly for improving the mechanical stability of the material, however, some studies demonstrated better results of the BCP containing high ratios of TCP (25-28). Arinzeh, et al (25) performed an in vivo study to determine the optimum ratios 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 the immunodeficient genetically disordered mice. The authors found that the BCP 20/80 had the better results when compared with the other proportions of the higher ratios of HA. Lomelino, et al (26) evaluated suitability of the BCP granules (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 of BCP granules alone were less than those of the other groups, 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/TCP including 30/70, 40/60 and 50/50 (28). 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 group of 50/50 followed by 30/70 and 40/60 respectively. Therefore, it implies that the BCP containing a higher ratio 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 (29) 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 the present study, BCP 30/70 was considered to be 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 at the temperature between 100-120°C, is appropriate for the melt blending with PCL and the monofilaments of PCL-BCP blends can be fabricated homogenously via an extruding machine (2). This study aimed to evaluate bioactivities of the PCL-BCP scaffolds which being composed of various ratios of PCL/ BCP for supporting growth and differentiation of the bone-forming cells. In addition, relations between the releasing ions from the scaffolds and responses of the cells were assessed and discussed.

Background

Physical characteristics of the PCL-BCP mMSMD scaffolds

The morphologies

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 (2). Regarding the previous study (30), the PCL-BCP scaffolds using the ratios of PCL: BCP = 80:20 (PCL-20%BCP) and 70:30 (PCL-30%BCP) has acceptable physical properties and good biocompatibility for using as bone substitutes. The architectures of the scaffolds consist of the large interconnecting pore system and high porosity (65.73 ± 5.02 %) which are considered to be appropriated for new bone regeneration (31-33) (Figure 1).

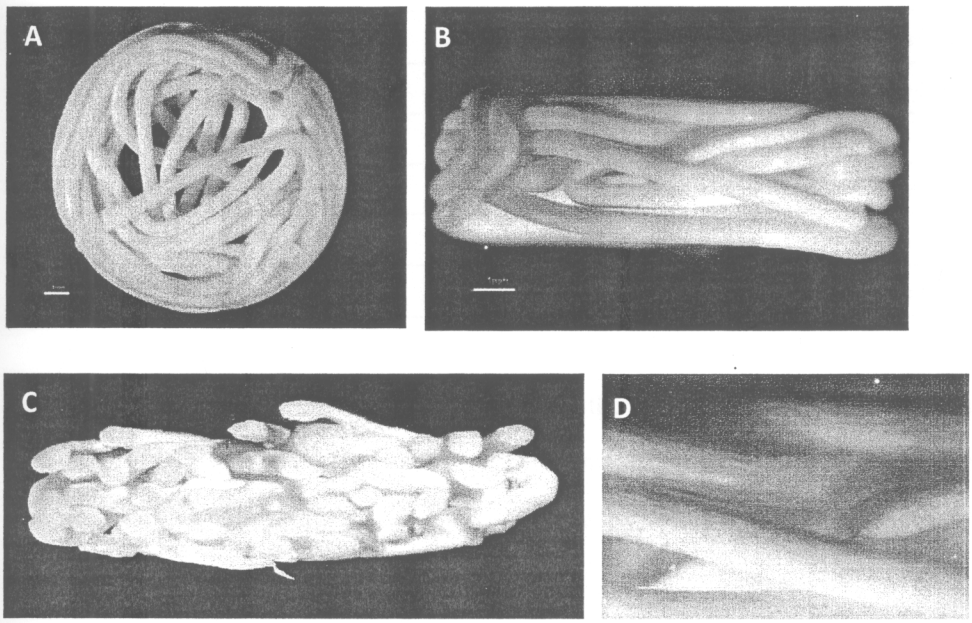


Figure 1 The stereomicroscope images demonstrate the morphologies of the PCL-BCP scaffold; A: superior view, B: lateral view, C: cross-sectional view and D: the magnified picture focuses on the rough surface architecture of the scaffold.

The mechanical properties

Regarding the previous study (30), the mechanical properties of the scaffolds were assessed using a universal testing machine. The result demonstrates that their mechanical properties are similar to those of the MSMD scaffolds as previously reported (3). The scaffolds have high elasticity and they can tolerate the compressive forces in the superior directions equal to the normal bite force. The profiles of the mechanical properties of the PCL-BCP mMSMD scaffolds, human bone, and the pure PCL MSMD scaffolds are comparatively demonstrated in table 1. The data shows that the compressive strength of the mMSMD scaffolds is comparable to that of the human cancellous bone. In addition, their properties are similar to those of the PCL MSMD scaffolds.

	Young's modulus (MPa)	Compressive strength (MPa)
Cortical bone	7000-30000	100-200
Cancellous bone	50-500	2-12
PCL MSMD scaffolds	13.05±0.7	2.78±0.04
PCL- 20%BCP mMSMD scaffolds	15.38±0.46	3.61±0.1
PCL- 30% BCP mMSMD scaffolds	17.72±3.61	3.83±0.19

Table 1. The table shows the mechanical profiles of the human bone (34), the pure PCL MSMD scaffolds (2, 3) and the PCL-BCP mMSMD scaffolds. The compressive strength of PCL-BCP mMSMD scaffolds is comparable to the human cancellous bone and there is no statistical difference of mechanical properties among the three types of scaffolds.

The degradation behaviors

Regarding the previous study (35), the degradability of the scaffolds was evaluated *in vitro*. The degradability test was done by immersing those scaffolds in the simulated body fluid (SBF) for 60 days, whilst, the *in vivo* experiment (pending for publication) was done by implanting those scaffolds subcutaneously in the back of male Wistar rats for 60 days. The result *in vitro* showed that the PCL-20%BCP scaffolds had slow degradation rate (weight loss = 1.91±0.47% on day 60). In contrast, the in vivo degradation rates of the scaffolds were rapidly increased with time (volume lost= 30.06±6.04% on day 60) which was significantly higher than *in vitro* ($p<0.05$). The slow degradation property of the PCL- BCP scaffolds is still an advantage because the strength of the scaffolds can be maintained as supportive structures over the entire periods of complete bone remodeling which usually takes around 6 months. In addition, their slow degradation could sustain longer releasing of calcium and phosphate ions, which are essential for bone regeneration.

The capability of release calcium and phosphate ions

Those ions are considered to be the very important factors for enhancing new bone formation supported by several previous studies (33, 36-40). Regarding the previous study(30), the PCL-BCP scaffolds could maintain releasing calcium and phosphate ions throughout 30 days when immersed in double distilled

water (dH_2O) (Figure 2). Although the maximum releasing of the ions was detected on day 7 and the rates of releasing decreased thereafter, the amount of those ions was still cumulatively increased with time.

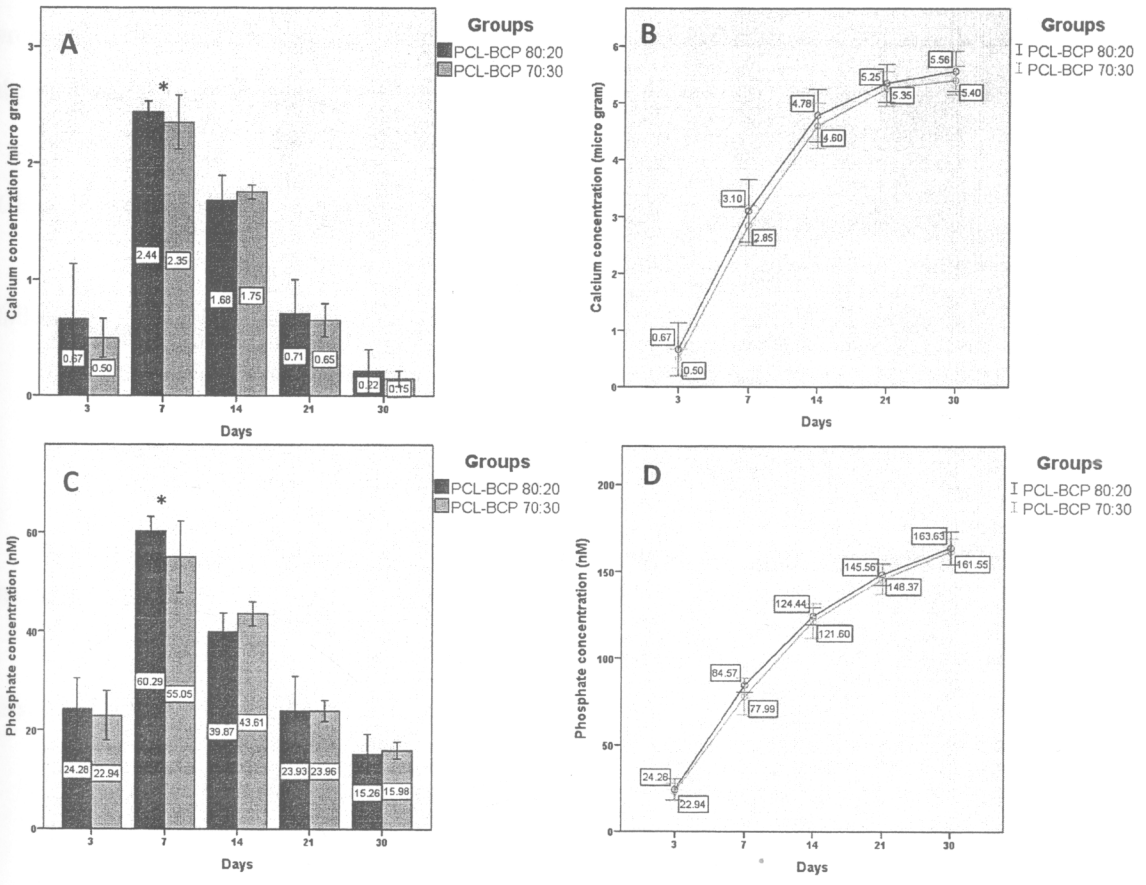


Figure 2 A and C; the graphs demonstrate the profiles of calcium and phosphate ions releasing in dH_2O over 30 days. The maximum release of both ions were detected on day 7 which was significantly greater than the other days (*; ANOVA, $p < 0.05$), and then, the release gradually decreased thereafter. B and D; the graphs demonstrate cumulative the release of the ions over 30 days. The accumulation of the releasing ions from the scaffolds increased with time.

Hypothesis

The PCL-BCP mMSMD scaffolds are biocompatible and they can sustain releasing of the essential calcium and phosphate ions which indirectly support growth and differentiation of the osteoblasts. The architectures of those scaffolds are suitable for proliferation and differentiation of the cells directly attaching on the scaffolds. In addition, the percentages of the BCP filler in those scaffolds would affect those properties.

Objectives of the study

The PCL-20%BCP and PCL-30%BCP mMSMD scaffolds were comparatively assessed as follows:

- Capability to release calcium and phosphate ions
- Effects of the releasing ions and the scaffold architectures on proliferation and differentiation of the osteoblast cells

Benefit of the study

To provide a scientific knowledge of the PCL-BCP mMSMD scaffolds being used for bone tissue engineering and used as the bone substitutes

Materials and Methods

Scopes of the study

The in vitro experiments were performed for evaluating the releasing profiles and the amounts of calcium and phosphate ions released from the PCL-BCP mMSMD scaffolds. The indirect cytocompatibility test was performed for investigating the effects of those ions on the proliferation and differentiation of the osteoblast cells (MC3T3-E1); by measuring the levels of specific markers of the cells. The direct cytocompatibility test was performed for measuring those markers from the cell-scaffold constructs to assess the capability of cells to growth and differentiate on the scaffold surfaces. The scaffolds as the following ratios of PCL: BCP were fabricated for the experiments; **group A;** 80:20 (PCL-20%BCP) and **group B;** 70:30(PCL-30%BCP), the positive control groups; **group C** using PCL: TCP= 80:20 (PCL-20%TCP) and **group D**=70:30 (PCL-30%TCP) and the negative control group; **group E** using PCL 100% (pure PCL).

Scaffold fabrication

The PCL-BCP scaffolds were fabricated using the mMSMD technique as follows. In brief, PCL pellets (Mn 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 the melting-extruding machine (2). The PCL-BCP monofilaments were made by extruding the PCL-BCP blend through the nozzle tip of the machine. Afterwards, the filaments were stretched to decrease their diameters and then they were stocked for fabricating the scaffolds. To fabricate each 3-D scaffold (Figure 3), 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, immersed into warm dH_2O. By using this technique, contacted surfaces of the filaments could be fused together and a 3-D scaffold (diameter: 11 mm, height: 3 mm) was built. The PCL-TCP and pure PCL scaffolds were fabricated by mixing of the PCL pellets and TCP particles (100% TCP, particle sizes< 75 μm, MTEC, Pathumthani, Thailand) or using 100% of the PCL pellets and their processing was the same as described above. Morphologies of the scaffolds of all groups were demonstrated in figure 1, 4 and 5. The scaffolds were sterilized using ethylene oxide gas 2 weeks prior to the experiments.

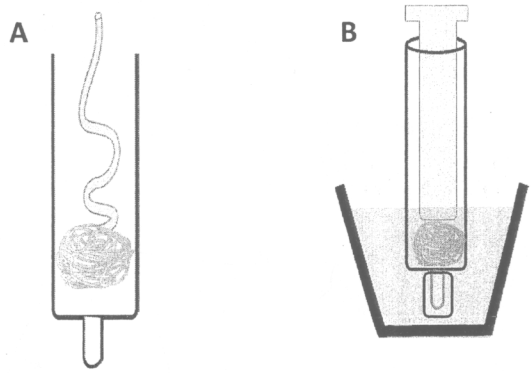


Figure 3 The fabrication process of the PCL-BCP scaffolds using the mMSMD technique; A: the PCL-BCP filament was put into a glass mold and compressed, B: the mold was immersed in warm dH_2O allowing the contact points of the filament to fuse together and form a 3-D scaffold.

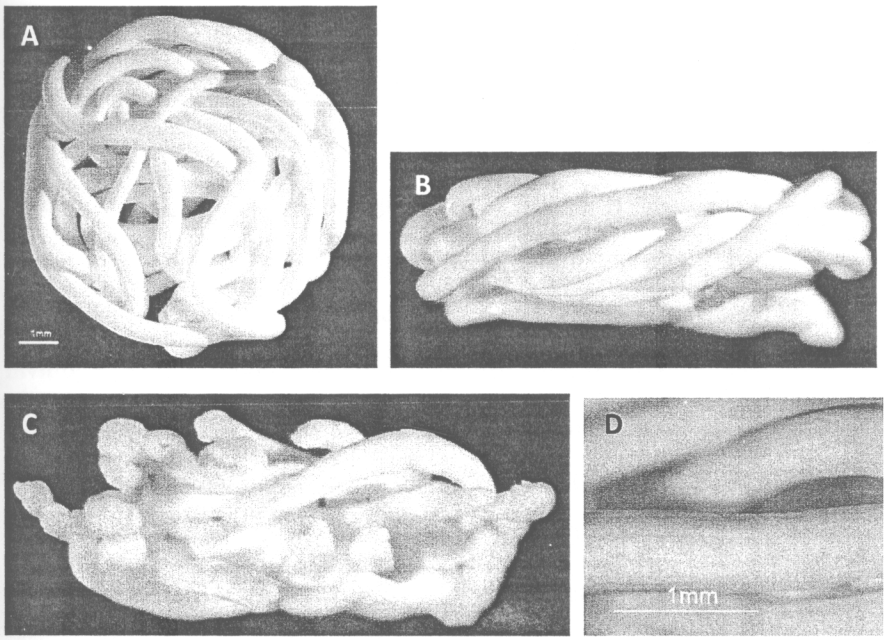


Figure 4 The stereomicroscope images demonstrate the morphologies of the PCL-TCP scaffold; A: superior view, B: lateral view, C: cross-sectional view and D: the magnified picture focuses on the rough surface architecture of the scaffold.

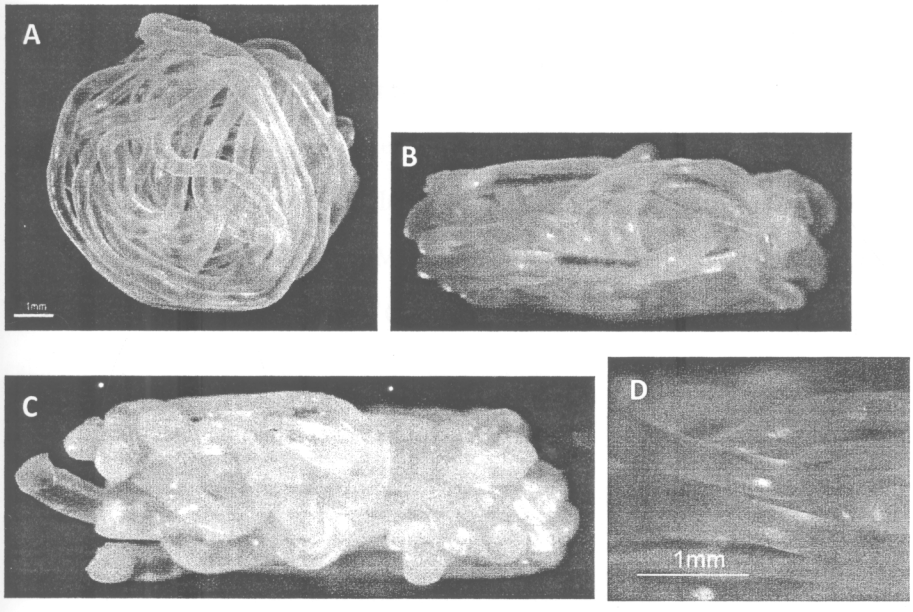


Figure 5 The stereomicroscope images demonstrate the morphologies of the pure PCL scaffold; A: superior view, B: lateral view, C: cross-sectional view and D: the magnified picture focuses on the scaffold surfaces.

Release of Calcium and Phosphate ions

The scaffolds of group A-D were left in proliferation culture medium [PR, 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 μg/mL fungizone (Gibco, Invitrogen, USA)] (n=25/group) for detecting the release of calcium and phosphate ions over 30 days. In brief, the scaffolds were immersed in 2 mL of the medium per well of a tissue culture plate, whilst, the proliferation medium and the osteogenic medium [OS, the proliferation medium supplemented with 10 mM β-glycerophosphate (Sigma, USA), 10^{-7} M dexamethasone (Sigma, USA) and 50 μM ascorbic acid-2 phosphate (Sigma, USA)] without the scaffolds were used as control groups. The plate was incubated at a constant temperature of 37°C, and on day 3, 7, 14, 21 and 30 thereafter, the scaffolds were moved to 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 to 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.

Cytocompatibility tests

1. Cell culture

Osteoblast cell lines (MC3T3-E1, subclone 4, ATCC, USA) were grown in the proliferation medium. The cells were cultivated in 5% CO_2 at 37 °C until reaching confluence, and then, subculturing was conducted. The cells between passages 3–6 were used for the experiments.

2. Indirect cytocompatibility test

The experiments were performed in order to evaluate an influence of the releasing ions from the scaffolds on proliferation and differentiation of the osteoblast cells. The schematic diagram of the tests was demonstrated in figure 6. On day 21, 14, 7 and 3 prior to the experiment, 1 x 10^4 cells were seeded into each well of the 48- well culture plates and then 200 µL of the PR medium were added (n=5/ group/ time point/ testing). The plates were left for 3 h in 5% CO_2 at 37°C to allow the cells to attach. Afterwards, the scaffolds of group A-D were immersed into each well and secured as close as possible to the bottom of the well. The wells of the PR and OS medium without the scaffolds were reserved for the control groups (n=5/medium/time point/testing). The plates were cultivated in 5% CO_2 at 37°C and the medium was changed every 3 days until the experiments.

2.1 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 the 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% CO_2 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.

2.2 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 µL 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 (OCN) assay. 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.

2.3 Matrix mineralization

The mineralization of the osteoblast cells were investigated by using Alizarin red S staining (AR) (41). On the day of the experiment, the cells were fixed with 4% formaldehyde for 10 min, and then, they were washed with distilled water and covered with a 2% Alizarin red S solution (Sigma, USA) for 5 min. After that, unincorporated dye were washed many times with distilled water and the stained spots of calcification were observed via a light microscope. To quantify the mineralization (42), the stained cells were incubated with 100 mM /L cetylpyridinium chloride (Sigma-Aldrich, USA) for 1 h in order to solubilize the calcium bound AR. The solutions were collected and read as units of AR released [1 unit is equivalent to 1 unit optical density at 570 nm measured using the micro-plate reader].

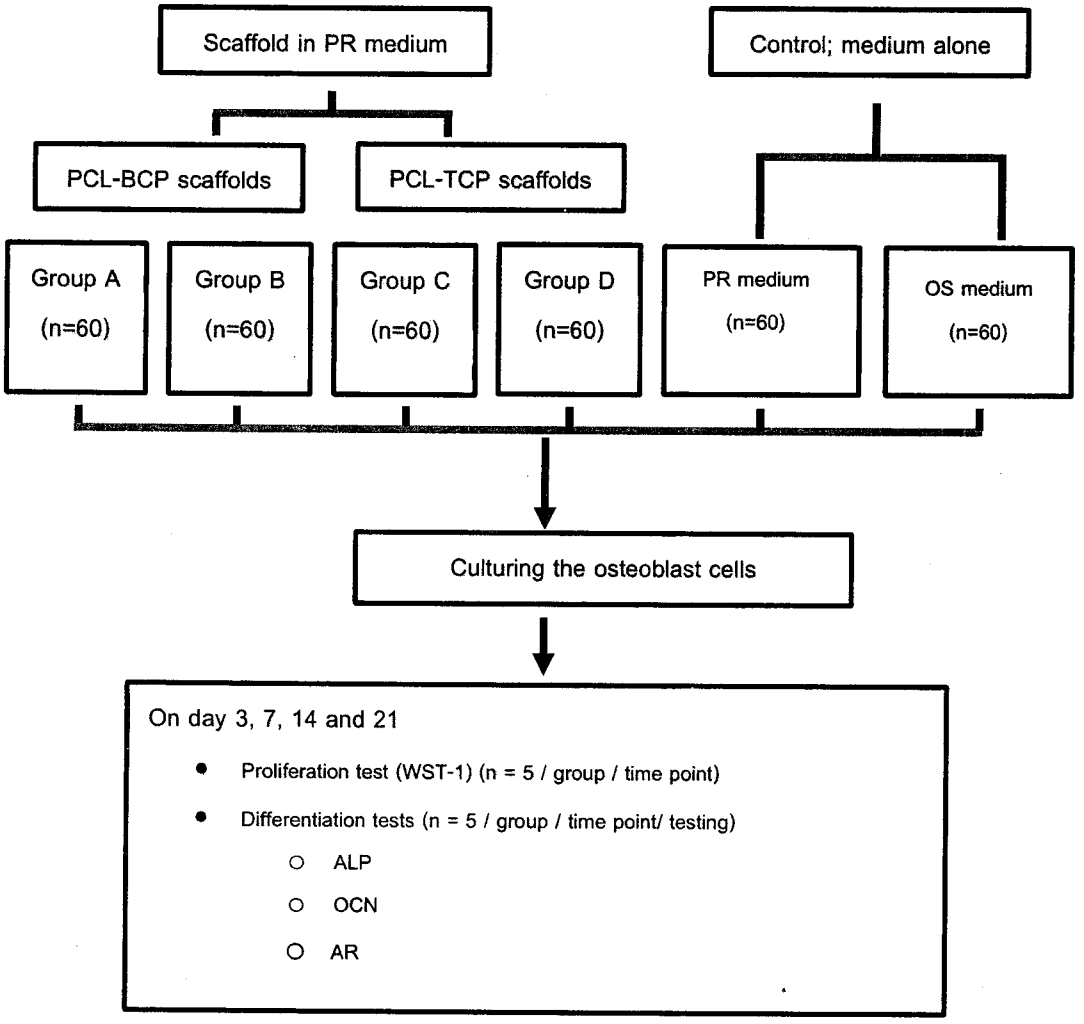


Figure 6 The indirect cytocompatibility test was done by assessing proliferation and differentiation of the osteoblast cells seeded on tissue culture plates and cultured in PR medium containing overlying scaffolds. On the culture days 3, 7, 14 and 21, proliferation and differentiation of those cells were evaluated by using WST-1 assay, measurements of ALP, OCN and AR. PR and OS mediums alone were used as the control groups.

3. Direct cytocompatibility tests

The tests were performed for evaluating the capability of osteoblast cells to growth and differentiate on the scaffold surfaces. The schematic diagram of this part was demonstrated in figure 7.

Preparing the cell-scaffold constructs

Prior to cell seeding, the scaffolds of group A, B and D were immersed in fresh PR medium for 24h. On day 30, 21, 14, 7 and 3 prior to the testing, the cell-scaffold constructs were made be seeding 2 x 10^5 osteoblast cells onto each scaffold using the static seeding method (n = 59/ group) (43). To ensure the seeding density of the cells throughout the scaffold, 1 x 10^5 cells (44) were seeded on each side of the scaffold and left in the incubator at 37°C, 95%CO_2 for 3h to allow the cell to attach. Afterwards, the scaffold was moved to the new well and the cells remaining in each well were trypsinized and counted to assess average amount of the cells, which fail to attach on the scaffolds. The constructs were cultivated in 500 µL of PR and OS medium in 5% CO_2 and 37 °C. The medium was changed every 3 days until the experiments.

3.1 Cell attachment and morphologies

The constructs cultured in PR medium for 3, 7 and 21 days were examined (n = 3/group/time point) via SEM (JEOL Ltd, Japan). The constructs were removed from the culture plates, rinsed with PBS, and then, fixed in 2.5% glutaraldehyde (Sigma-Aldrich, USA) in PBS for 2 h. After that, they were dehydrated in ethanol series of 50–100% and coated with gold-palladium. Characteristics of the cells in the constructs were descriptively assessed.

3.2 Cell proliferation

Amounts of the cells in the constructs on the culture-day 3, 7, 14, 21 and 30 were assessed using WST-1 assay as previously described (n = 5/group/time point).

3.3 Cell differentiation

On the day of the experiment, the constructs were moved to the new wells and washed two times in PBS. Afterwards, 200 µL of 1% Triton X-100 in PBS were added to each well, and then, the constructs were minced into small pieces. The cells were lysed by freezing and thawing for three cycles. The mixtures were transferred into the micro-centrifuge tubes and centrifuged at 2000 x g for 10 min. The supernatants were collected as the cell lysis solutions and they were kept in there at -80 °C for the analysis of total cellular protein content, ALP and OCN as previously described.

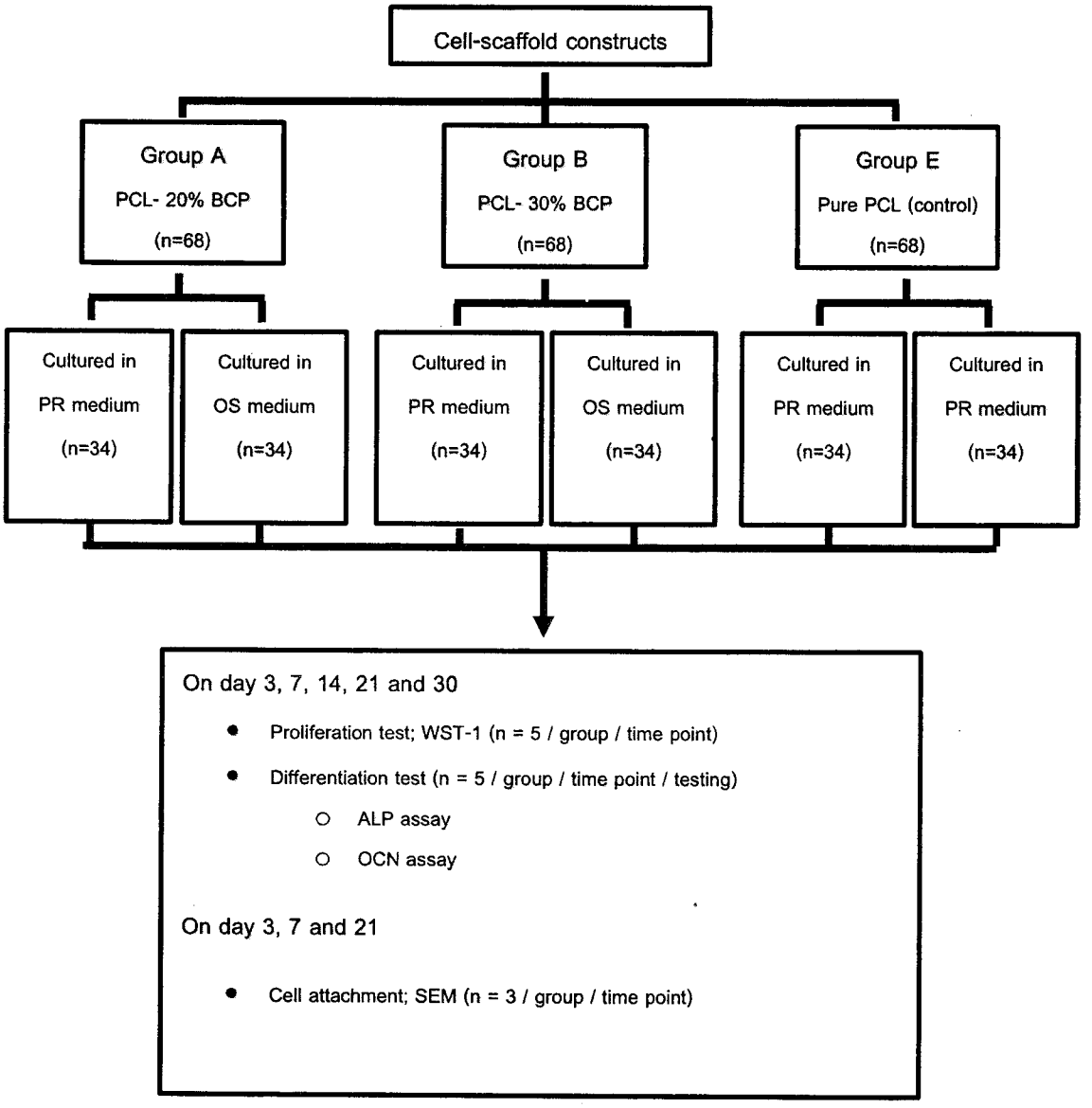


Figure 7 The diagram of the direct cytocompatibility test. Assessment of growth and differentiation of the osteoblast cells in the cell-scaffold constructs cultured in PR and OS mediums were evaluated over 30 days.

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 calcium and phosphate ions as well as the parameters of cytocompatibility tests among the groups. 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 amounts of calcium and phosphate ions released from the scaffolds were demonstrated in figure 8. The results demonstrated that the scaffolds of group A-D could release those ions over the observation periods when immersed in the PR medium and there was no significant difference among the groups over the time points (ANOVA, $p > 0.05$). It was noted that the releasing concentrations of the experiment groups on day 7 were significantly increased greater than other days (ANOVA, $p < 0.05$), and then, they decreased thereafter. However, the cumulative data demonstrated that the accumulation of those ions continued increasing with time. There was no significant difference of the cumulative concentrations of calcium ion among group A-D in every time point, whilst, those of phosphate ions of group A and C were significantly higher than group B and D in every time point.

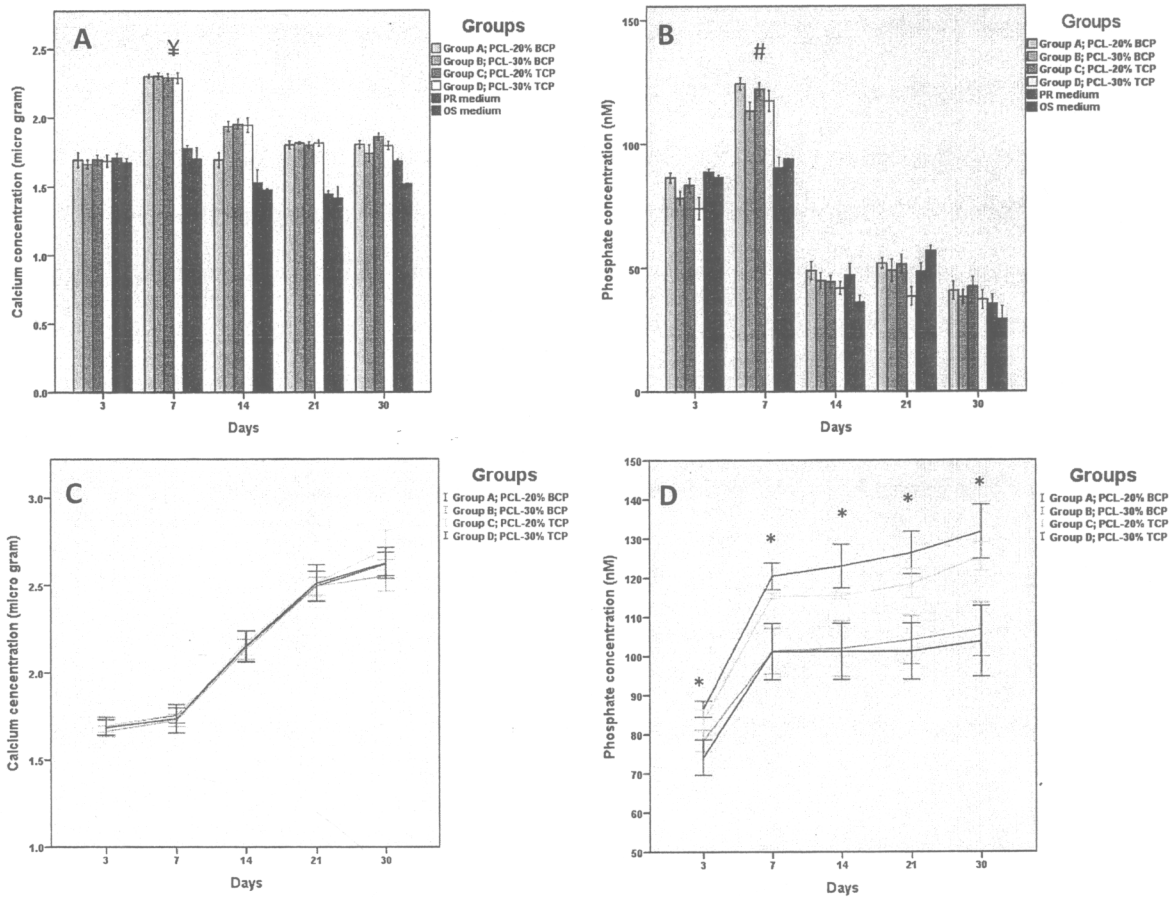


Figure 8 A and B; The graphs demonstrate the profiles of calcium and phosphate ions released from the scaffolds over 30 days. The maximum release of both ions in all groups were detected on day 7, which was significantly greater than the other days (¥, #; ANOVA, $p < 0.05$) and the releasing decreased thereafter. C; Accumulative release of calcium ion of all groups rapidly increased after day 7 until day 21, and then, the releasing rates slowed down thereafter. No significant difference was found among the groups over the time points (ANOVA, $p > 0.05$). D; Accumulative release of phosphate ion rapidly increased from day 3 to day 7, and then, the rates slowed down thereafter. It was found that the cumulative concentrations of group A and C were significantly greater than those of group B and D in every time point (*; ANOVA, $p < 0.05$).

Indirect cytocompatibility

Cell proliferation

The amounts of the viable cells of all groups over the observation period were demonstrated in figure 9. The cells of group A-D slightly decreased on day 7, and then increased thereafter to reach the maximum growth on day 21. The highest cell numbers on day 21 was detected in group B which was significantly higher than group A and control groups (ANOVA, $p < 0.05$), but, it was not significantly higher than group C and D (ANOVA, $p > 0.05$). In PR group, the maximum growth of the cells was detected on day 14 (significantly higher than other groups, ANOVA, $p = 0.00$), and then, it slightly decreased on day 21. However, on day 21, the viable cell numbers in PR and OS group were significantly less than those of the experiment groups (ANOVA, $p < 0.05$).

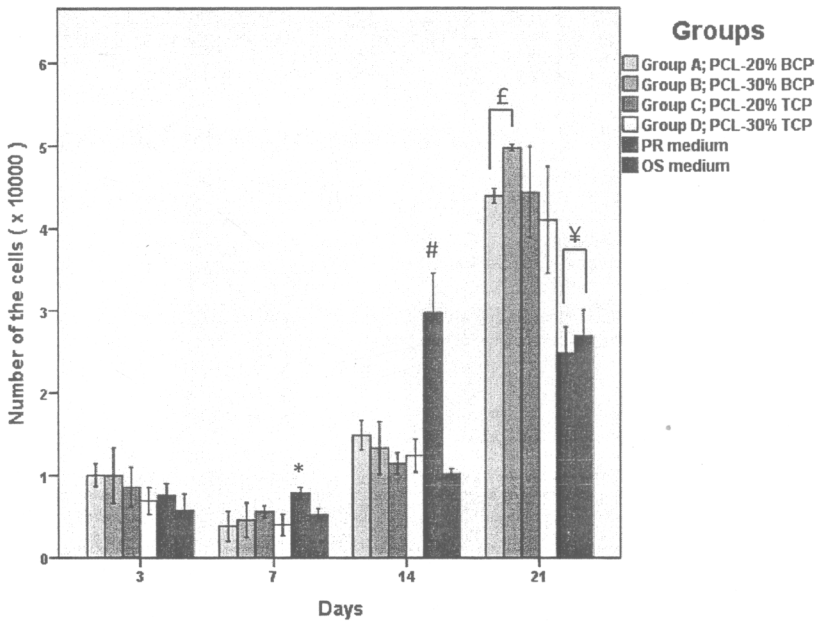


Figure 9 The bar graphs show that amounts of the cells in group A-D and in OS medium slightly decreased on day 7, and then, remarkably increased on day 14. On day 21, the amounts of the cells in those groups reached their maximum levels. The cells cultured in PR medium continued increasing since day 3 and reached the maximum level of day 14 (* and # = significantly greater than group A-D, ANOVA; $p < 0.05$), then, it decreased thereafter. On day 21; ¥ = PR and OS groups were significantly less than group A-D, ANOVA; $p < 0.05$ and £ = group B was significantly higher than group A, ANOVA; $p = 0.00$.

ALP activities

The levels of ALP were demonstrated in figure 10. It was found that the profiles of ALP in group A and D were similar over the observation period. Those profiles increased with times and reach the maximum levels on day 21. At that time, the activity of group A was significantly higher than the other groups (ANOVA, $p = 0.00$). The activity of group B was significantly higher than the other groups on day 3 (ANOVA, $p = 0.00$), and then, it decreased and became stable on the following days. The ALP of group C seemed to be stable over the observation periods. On day 14, the ALP of OS medium group was significantly higher than the other groups (ANOVA, $p = 0.00$), and then, it remarkably decreased on day 21.

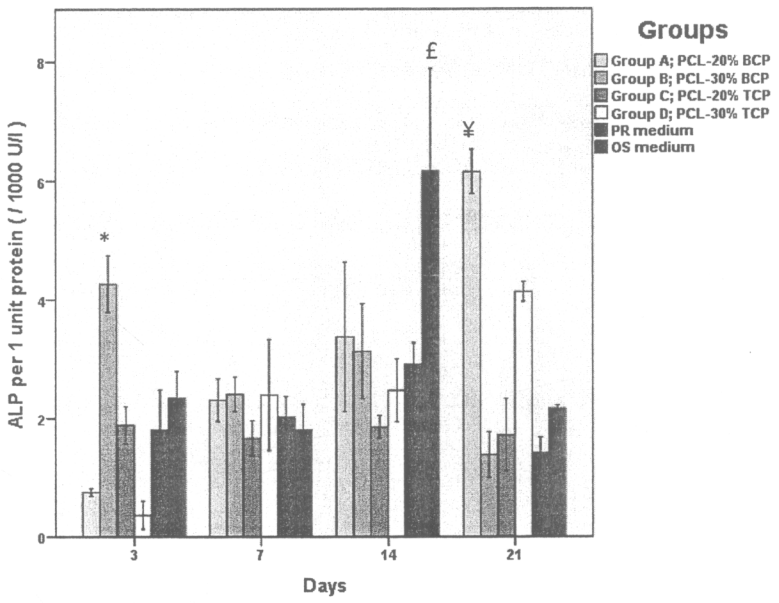


Figure 10 The graphs demonstrate the ALP activities of group A-D and the control groups of the medium alone over 21 days. The activity of group A was significantly higher than the other groups on day 21(¥; ANOVA, $p = 0.00$). The activity of group B was significantly higher than the other groups on day 3 (*; ANOVA, $p = 0.00$). The activity of the OS medium group was significantly greater than the other groups on day 14 (£; ANOVA, $p = 0.00$)

OCN

The OCN levels were demonstrated in figure 11. The OCN of all groups can be early detected since day 7. The levels of group A, B, and the OS medium group seemed to be stable over the first 14 days, and then increased on day 21. In contrast, those of group C and D seemed to be stable over the observation period. It was noted that the OCN levels of the control groups were less than those of group A and B since day 14, but, they were less than those of group C in every time point. For the experiment groups, on day 21, the highest OCN level was detected in group A followed by group B, C and D respectively (not significantly difference among the groups, ANOVA, $p > 0.05$).

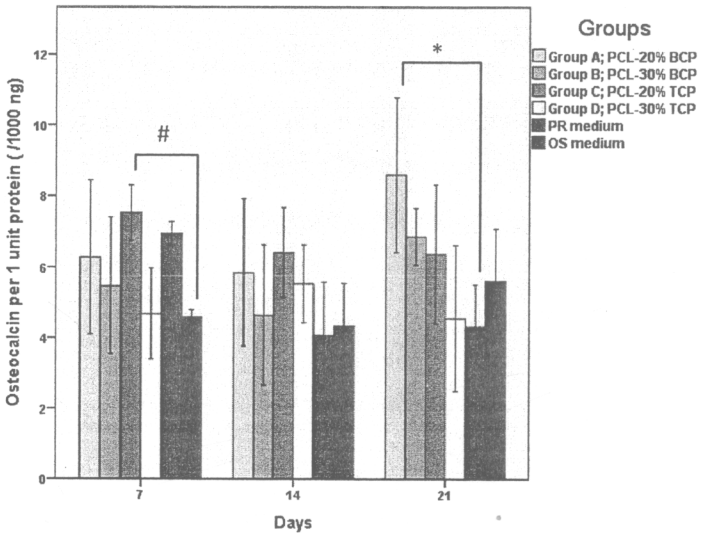


Figure 11 The graphs demonstrate the levels of OCN over 21 days. The level of group A had the highest concentration on day 21 (significantly higher than the PR medium group, *; ANOVA, $p = 0.00$). The level of group C was higher than those of the control groups in every time point (significantly higher than the OS medium group on day 7, #; ANOVA, $p = 0.02$).

AR staining

The solubilized AR staining of group A-D and the control groups over 21 days were demonstrated in figure 12. It was found that the levels of AR in group A-D seemed to be stable during the first 14 days, and then, they rapidly increased on day 21 (significantly higher than the PR medium group, but, significantly less than the OS medium group, ANOVA, $p < 0.05$). The levels in the OS medium group increased with time (significantly greater than the other groups since day 7), whilst, the levels in the PR medium group seemed to be stable over the observation period.

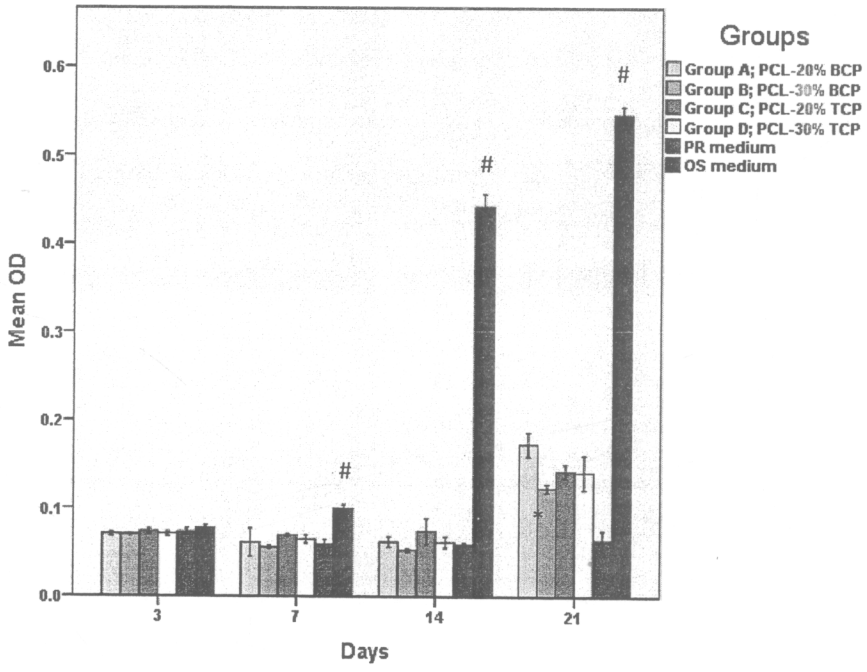


Figure 12 The graphs demonstrate the amounts of the solubilized AR over 21 days. The AR levels of group A-D were significantly higher that of the PR medium group on day 21 (*=significantly less than the other groups; ANOVA, $p < 0.05$). The AR levels in the OS medium group were significantly higher than the other groups since day 7 (#; ANOVA, $p < 0.05$).

Direct cytocompatibility tests

Cell attachment and morphologies

The SEM pictures in figure 13-15 demonstrated the behaviors of the osteoblasts in the cell-scaffold constructs of group A, B, and E when they were cultured in PR and OM mediums. It was found that the cells could attach and grow well throughout the scaffold surfaces of all groups. Since day 14, the cells grew and formed multilayer cell-sheets covering the entire surfaces. Mineralization nodules could be observed in all groups when cultured in OS medium since day 14.

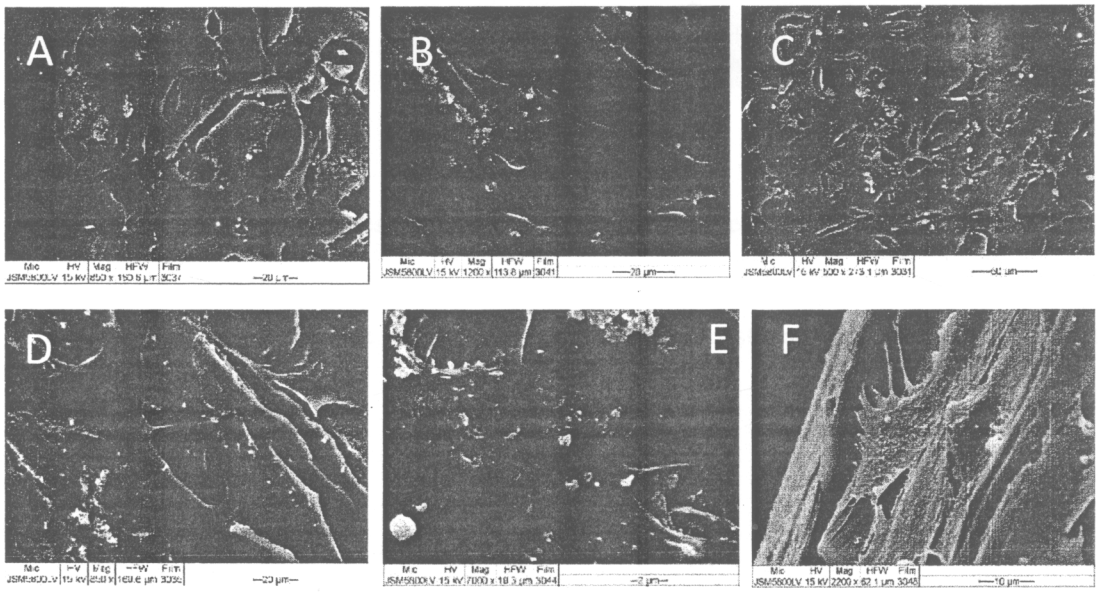


Figure 13 The SEM images of group A, B and E on the culture day 3; A-C: cultured in PR medium, D-F: cultured in OS medium. The osteoblast cells spread their cytoplasmic processes attaching well to the scaffold surfaces.

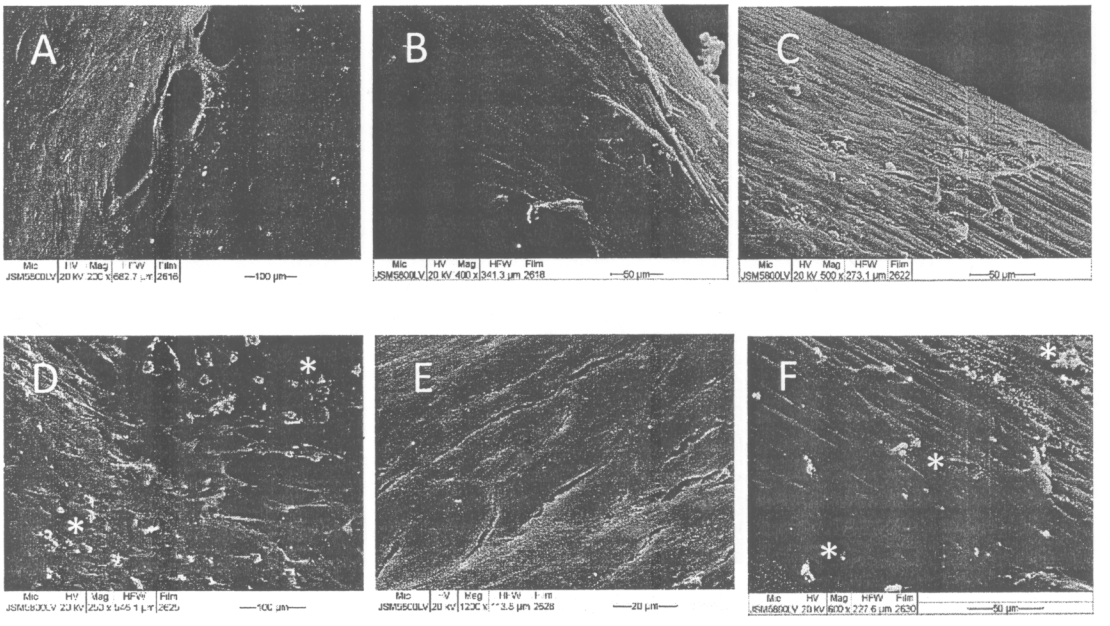


Figure 14 The SEM images group A, B and E on the culture day 14; A-C: cultured in PR medium, D-F: cultured in OS medium. Dense multilayer cell-sheets throughout the scaffold surfaces of all groups were seen. Mineralized nodules were detected in some areas (*) when the constructs were cultured in the OS medium.

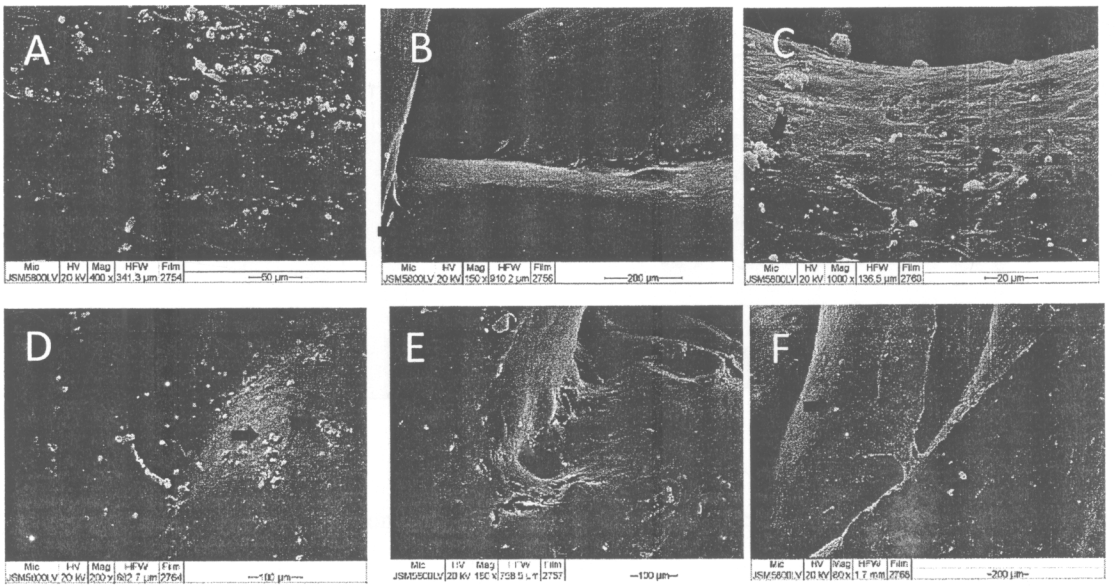


Figure 15 The SEM images group A, B and E on the culture day 21; A-C: cultured in PR medium, D-F: cultured in OS medium. Denser cell-sheets covered the entire scaffold surfaces of all groups and the morphologies of the cells were difficult to identify. Mineralized nodules were seen in some areas of all groups when cultured in both mediums (arrows).

Cell proliferation

The amounts of the viable cells in the cell-scaffold constructs were demonstrated in figure 16. In the PR medium, the amounts of the cells of group A increased with time and reached the maximum growth on day 30 (significantly higher than the other days, ANOVA, $p = 0.005$), whereas, those of group B reached the maximum growth on day 14, and then, decreased thereafter. The growth of group E increased until day 14, and then, decrease on day 21. After that, the growth increased to the maximum level on day 30. It was noted that the growth of the cells in group A and B were greater than those in group E in every time point. In the OS medium, the amounts of the cells of group A significantly increased on day 7, and then, rapidly decreased on day 14 (ANOVA, $p = 0.004$ and 0.017 respectively). The growth reached the maximum level on day 21(significantly increased from day 14, ANOVA, $p = 0.003$). The growth of the cells in group B gradually increased with time in the first 14 days, then, they slightly decreased on day 21 and reached the maximum level on day 30 (ANOVA, $p = 0.085$). Similar to the cells cultured in the PR medium, the amounts of the viable cells of group A and B were higher than group E in every time point, except on day 21 when group E were slightly higher than group B.

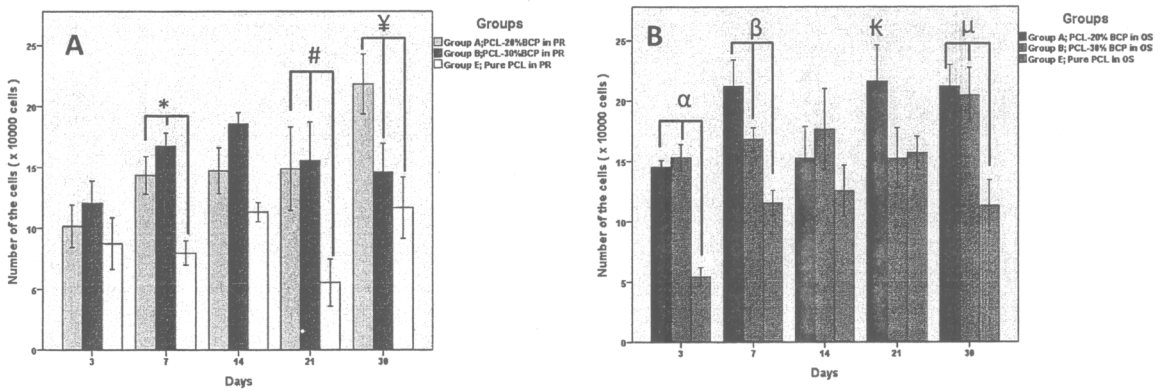


Figure 16. The graphs demonstrate the growth profiles of the cells in the cell-scaffold constructs of group A, B, and E cultured in the different mediums over 30 days. A; In the PR medium, the viable cells of group A and B were greater than those of group E in every time point (significantly different on day 7, 21 and 30; *, #, and ¥; ANOVA, $p < 0.05$). B; In the OS medium, the viable cells of group A reached the maximum level on day 21 (significantly higher than day 14, ₭; ANOVA, $p = 0.003$). The viable cells of group B slightly increased with time and then reached the maximum level on day 30. The amounts of the cells of group A and B were significantly higher than those of group E on day 3, 7 and 30 (α, β, μ; ANOVA, $p < 0.05$).

ALP activities

The ALP activities of the cells in the constructs were demonstrated in figure 17. In the PR medium, the highest ALP activity of group A was detected on day 3, and then, they decreased to the lowest level on day 21, but, remarkably increased on day 30. The ALP of group B increased to the maximum level on day 7, and then, significantly decreased to the lowest level on day 14, but, remarkably increased on day 30. The activity of group E seemed to be stable over the observation period. It was noted that the ALP activities of group A and B were greater than those of group E in every time-point and there was no significant difference between group A and B over the observation period. In the OS medium, the activities of group A and B increased with time to reach their maximum levels on day 14 and they seemed to be stable thereafter. On day 21, the activity of group B was significantly higher than group A. It was noted that the activities of group A and B were higher than those of group E over the observation period (significantly different from since day 7, ANOVA, $p < 0.05$)

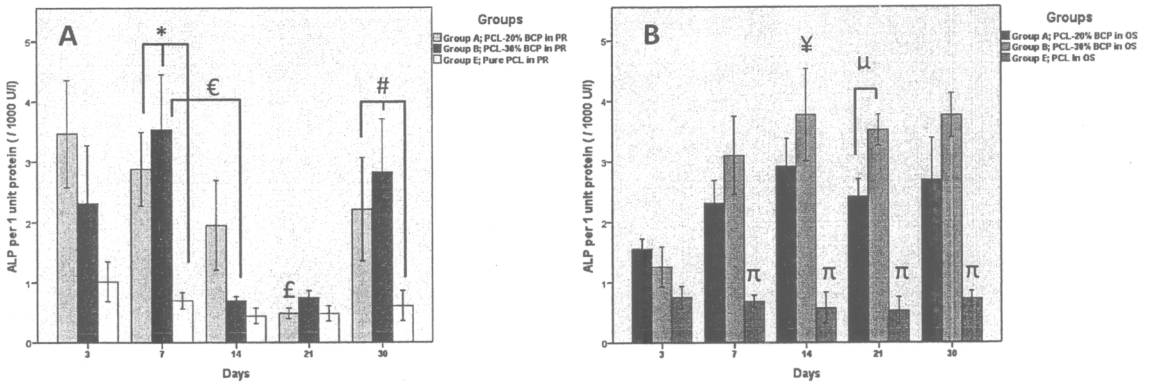


Figure 17 The graphs demonstrate the ALP activities of cells in the constructs of group A, B and E cultured in the PR and OS mediums over 30 days. A; In the PR medium, the ALP activity of group A on day 21 was significantly lower than other days (£; ANOVA, p =0.008). The activity of group B significantly decreased on day 14 (€; ANOVA, p = 0.00). The activities of group A and B were significantly higher than those of group E on day 7 and 30 (*, #; ANOVA, $p < 0.05$). B; In the OS medium, the ALP activity of group A and B reached the maximum level at day 14 (¥; ANOVA, $p<0.05$)), and the activity of group B was significantly higher than group A on day 21 (μ; t-test, p = 0.001). Since day 7, the activities of group E were significantly less than those of group A and B (π; ANOVA, $p < 0.05$).

OCN

The expression of OCN of the cells in the constructs of group A, B, and E, were demonstrated in figure 18. In the PR medium, the OCN levels of group A and B can be detected since day 7, they were stable until day 21, and then, significantly increased to the maximum levels on day 30 (ANOVA, $p < 0.05$). In contrast, the OCN levels of group E seemed to be stable over the observation period. Except day 14, the levels of group A and B were higher than those of group E. In the OS medium, the profiles of the OCN levels of group A and B were similar to those in the PR medium. The OCN level of group A and B were stable until day 21and reached the maximum levels on day 30 (ANOVA, $p < 0.05$). The OCN levels of group E gradually increased since day 14 and reached the maximum level on day 30 (ANOVA, p = 0.016). The levels of group E were less than those of group A and B on day 7, 14 and 30.

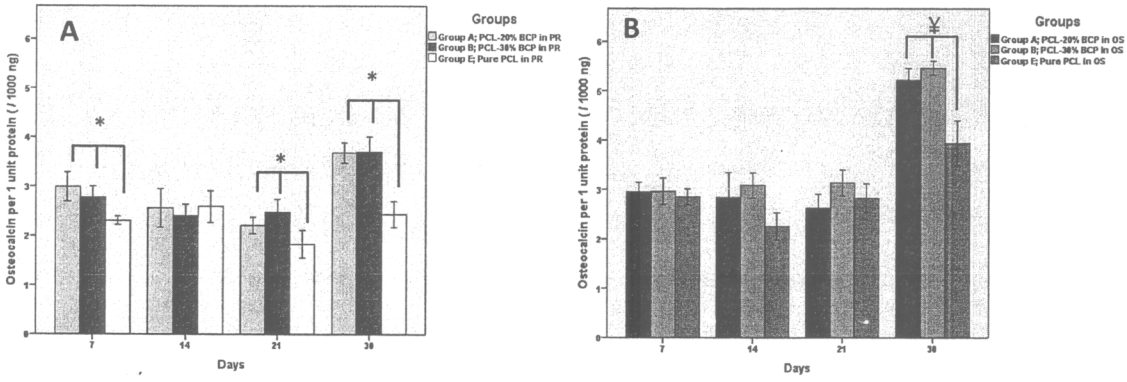


Figure 18 A; The graphs demonstrate the OCN levels of the cell-scaffolds constructs cultured in PR medium over 30 days. The levels of group A and B were stable until day 21, and then, increased to the maximum level on day 30. The levels of group A and B were greater than those of group E on day 7, 21 and 30 (*; ANOVA, $p < 0.05$) B; In the OS medium, the levels of group A and B were stable until day 21, and then, significantly increased on day 30. The levels of group A and B were significantly greater than that of group E on day 30 (¥; ANOVA, $p<0.05$).

Discussion

This study was performed in order to evaluate direct and indirect effects of the bioactivities of the PCL-BCP mMSMD scaffolds on osteoblastic responses. The two different ceramic fillers in the PCL-based scaffolds were used including BCP which composing of the static phase of HA at 30% and the soluble phase of TCP at 70% and 100% TCP filler. By comparing to the PCL-TCP scaffolds and the pure PCL scaffolds, therefore, the actions of the BCP can be elucidated. Two main experiments were performed including direct and indirect cytocompatibility tests to confirm the actions of BCP filler as well as the effects of the architectures of the mMSMD scaffolds on growth and differentiation of the cells. For the indirect cytocompatibility test, the releasing profiles of calcium and phosphate ions were assessed when the scaffolds were immersed in the proliferation medium. The PCL-TCP scaffolds were used for comparing the amounts of the releasing ions with the PCL-BCP scaffolds, whilst, the osteogenic medium and the proliferative medium alone were served as the positive and negative control groups respectively. In addition, measuring proliferation and differentiation of the osteoblast cells cultured in the mediums of each group is performed to assess the indirect effects of the releasing ions on the cells. By measuring the amounts of the ions in the medium instead of dH_2O, their total concentrations that truly influencing proliferation and differentiation of the osteoblast cells are assessed. In addition, those concentrations can be compared with those in an osteogenic medium which considered to be the optimum concentrations for inducing the differentiation of the cells. In this study, the entire phases of the osteoblastic differentiation were assessed including measuring ALP for the early differentiation and OCN for the late differentiation. In addition, the AR was used for assessing the mineralized nodule formation to infer that the cultured cells could produce bone-like matrix. The direct cytocompatibility test was performed to evaluate the influence of the scaffold morphologies rather than the releasing ions on the cellular responses. Therefore, the PCL-TCP scaffolds were not used for comparing with the PCL-BCP scaffolds and the pure PCL scaffolds without bioactive filler were used as the negative control instead. In this experiment, growth and differentiation of the cells attaching on the surfaces of the scaffolds were evaluated, except, measuring the solubilized AR. The matrix mineralization was not assessed in order to avoid its false positive from absorbing excessive staining of the scaffolds and staining carbonate apatite layers (6) which occasionally occurred on the surfaces of the scaffolds when immersed in the culture solutions.

Regarding the results, the PCL-BCP scaffolds containing the BCP filler of 20% and 30% could sustain release of calcium and phosphate ions throughout 30 days. Interestingly, there was no statistical difference of the amounts of the calcium ions and their releasing profiles among the groups of the PCL-BCP and the PCL-TCP scaffolds. Moreover, the cumulative concentrations of the phosphate ions released from the PCL-20%BCP scaffolds and the PCL-20%TCP scaffolds were significantly higher than those from the scaffolds using the fillers of 30%. Theoretically, TCP can be solubilized easier than BCP, hence, at the same ratios, the PCL- TCP scaffolds would release more calcium and phosphate ions into the medium when compared with the PCL-BCP scaffolds. It turned out that there was no statistical difference of the releasing concentrations of the calcium ions among the groups, whilst, the releasing profiles of the phosphate ions were 20%BCP>20%TCP>30%of TCP and 30%BCP. Therefore, it implies that using of TCP filler and BCP filler has the similar capacities of releasing calcium and phosphate ions and increasing the fillers up to 30% as a limitation would not enhance the release. These phenomena could be explained that the saturated solution would slow down the release of the ions and it affected the releasing phosphate ion more than the calcium ions particularly in the culture medium. In addition, the amounts of the fillers of 20% and 30% are slightly different leading to a difficulty in explain the phenomena.

Some previous studies demonstrated the effect of calcium and phosphate ions on behaviors of bone cells (37, 38, 45, 46). For the effect of calcium ion, Maeno, et al (46) 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 (38) 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 (45) 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 (37) 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 increase of collagen content and calcification of the cultures. However, addition of

high concentration of phosphate ranging from 5 to 7mM was reported to induce in vitro osteoblast apoptosis and non-physiological mineral deposition (47). The optimal concentrations of calcium and phosphate ions may vary according to the cellular stages of proliferation and differentiation and there is no optimal concentration that universally stimulate the cells toward successful osteogenesis (48). Regarding the results of our study, the accumulative concentrations of calcium and phosphate ions in the culture medium are demonstrated in table 2.

Day	Calcium ion (mM)				Phosphate ion (mM)			
	20%BCP	30%BCP	20%TCP	30%TCP	20%BCP	30%BCP	20%TCP	30%TCP
3	0.85	0.83	0.85	0.84	0.086	0.078	0.084	0.074
7	0.88	0.86	0.87	0.87	0.120	0.101	0.115	0.101
14	1.07	1.06	1.08	1.07	0.122	0.102	0.115	0.101
21	1.25	1.24	1.26	1.25	0.126	0.104	0.119	0.101
30	1.31	1.27	1.35	1.31	0.131	0.106	0.125	0.104

Table 2 The table shows the average accumulative concentrations (mM) of calcium and phosphate ions in the culture medium.

Although the data shows that the concentrations of those ions are lower than the optimum ranges for supporting the functions of osteoblasts, the responses of the osteoblast cells seemed to relate to the releasing ion concentrations and the ratios of the ceramic fillers in the scaffolds. The burst release of the phosphate ion on day 7 related to the decrease of the proliferation of the cells, whilst, that of calcium ion after day 7 related to the increase of their proliferation. It implies that the concentrations of phosphate ion on day 7 produced an inappropriate environment for the growth of the forming cells, on the other hand, the decrease of the releasing rate thereafter would promote their proliferation. In contrast, the increase of calcium ion would promote the cell proliferation since day 7. When compared the viable cell number between the PCL-BCP groups and the PCL-TCP groups, it was found that there was no statistical difference among the groups. Therefore, the solubilization of the TCP filler does not have any advantage for promoting the growth of the osteoblasts when compared with the BCP filler. Regarding the aspects of the cell differentiation, it was found that the groups of PCL-BCP scaffolds seem to have the better result in stimulating the ALP activity of the cells over the observation period when compared with the PCL-TCP scaffolds, especially the activities in the PCL-20%BCP group which increasing with time and being remarkably greater than the other groups on day 21. The indicative markers of the late differentiation of the cells including OCN and AR indicated that the groups of PCL-20%BCP and PCL-

20%TCP scaffolds were dominant. The levels of OCN in both groups were detected at the higher levels since day 7 when compared with the other ratios. In addition, the level in PCL-20%BCP group remarkably increased to the maximum level on day 21 which was higher than the other groups. Similarly, the profiles of the solubilized AR indicated that both scaffolds could indirectly enhance the degrees of mineralization of the osteoblasts greater than the other groups. Therefore, the composition of the PCL-based scaffolds using 20% of BCP and TCP fillers would have some advantages for promoting new bone regeneration and the extending experiments for evaluating those effects should be performed both in vitro and in animal models.

Regarding the direct cytocompatibility experiments, the SEM images demonstrated that the mMSMD scaffolds could support attachment, proliferation as well as differentiation of the osteoblast cells regardless of the compositions of materials. However, it was found that the PCL-BCP scaffolds had the better results for those purposes when compared with the pure PCL scaffolds whether cultured in PR or OS mediums. For the cell proliferation, growth of the cells in the PCL-30%BCP scaffolds was faster than that in the PCL-20%BCP scaffolds when the constructs were cultured in the PR medium. In contrast, in the OS medium, the cells in the PCL-20%BCP scaffolds had the faster result. For the ALP activities, the cells in the PCL-20% scaffolds expressed ALP earlier than those in the PCL-30%scaffolds when the constructs were cultured in the PR medium. However, the cells of both groups had the same profiles of the ALP activities when cultured in the OS medium. For the levels of OCN, the cells of both groups when cultured in both mediums had similar profiles of the expression. It was noted that the cells in the PCL-20%BCP scaffolds had the better osteoblastic differentiation due to the higher levels of ALP and OCN in the earlier days of the experiments when compared with those in the PCL-30%BCP scaffolds even the constructs were cultured in the medium without supporting substances for the cell differentiation. However, the direct cytocompatibility of the PCL-20%TCP scaffolds is not yet evaluated and compared with those of the PCL-20%BCP scaffolds. Therefore, that extending experiments should be done in the future to confirm that which one is the optimum filler for the PCL-based mMSMD scaffolds.

Conclusion

The technique of mMSMD is very practical to fabricate PCL- ceramics 3-D scaffolds. The architectures of the scaffolds are appropriate for supporting attachment and functions of osteoblasts. The calcium and phosphate ions, which are essential for process of new bone formation, can sustain their release from the scaffolds over the period of bone formation. In addition, the release of those ions would influence the activities of the cells. The PCL-20%BCP scaffolds have the better indirect and direct support to the entire phases of the osteoblastic differentiation when compared with the PCL-30%BCP scaffolds.

References

1. Salgado AJ, Coutinho OP, Reis RL. Bone tissue engineering: state of the art and future trends. MacromolBiosci. 2004;4(8):743-65.

2. Thuaksuban N, Nuntanaranont T, Pattanachot W, Suttapreyasri S, Cheung LK. Biodegradable polycaprolactone-chitosan three-dimensional scaffolds fabricated by melt stretching and multilayer deposition for bone tissue engineering: assessment of the physical properties and cellular response. Biomed Mater. 2011;6(1):015009.

3. Thuaksuban N, Nuntanaranont T, Suttapreyasri S, Pattanachot W, Sutin K, Cheung LK. Biomechanical properties of novel biodegradable poly epsilon-caprolactone-chitosan scaffolds. J Investig Clin Dent. 2013;4(1):26-33.

4. Thuaksuban N, Nuntanaranont T, Suttapreyasri S, Boonyaphiphat P. Repairing calvarial defects with biodegradable polycaprolactone-chitosan scaffolds fabricated using the melt stretching and multilayer deposition technique. BiomedMaterEng. 2015;25(4):347-60.

5. Engelberg I, Kohn J. Physico-mechanical properties of degradable polymers used in medical applications: a comparative study. Biomaterials. 1991;12(3):292-304.

6. Lei Y, Rai B, Ho KH, Teoh SH. In vitro degradation of novel bioactive polycaprolactone—20% tricalcium phosphate composite scaffolds for bone engineering. Mater Sci Eng C. 2007;27(2):293-8.

7. Rai B, Teoh SH, Ho KH. An in vitro evaluation of PCL-TCP composites as delivery systems for platelet-rich plasma. J Control Release. 2005;107(2):330-42.

8. Schantz JT, Hutmacher DW, Lam CX, Brinkmann M, Wong KM, Lim TC, et al. Repair of calvarial defects with customised tissue-engineered bone grafts II. Evaluation of cellular efficiency and efficacy in vivo. Tissue Eng. 2003;9 Suppl 1:S127-39.

9. Tay BY, Zhang SX, Myint MH, Ng FL, Chandrasekaran M, Tan LKA. Processing of polycaprolactone porous structure for scaffold development. J Mater Process Technol. 2007;182(1-3):117-21.

10. Woodruff MA, Hutmacher DW. The return of a forgotten polymer—Polycaprolactone in the 21st century. Prog Polym Sci. 2010;35(10):1217-56.

11. Lam CX, Hutmacher DW, Schantz JT, Woodruff MA, Teoh SH. Evaluation of polycaprolactone scaffold degradation for 6 months in vitro and in vivo. J Biomed Mater Res A. 2009;90(3):906-19.

12. Dorozhkin SV. Bioceramics of calcium orthophosphates. Biomaterials. 2010;31(7):1465-85.

13. Bagambisa FB, Joos U, Schilli W. Mechanisms and structure of the bond between bone and hydroxyapatite ceramics. J Biomed Mater Res. 1993;27(8):1047-55.

14. Tracy BM, Doremus RH. Direct electron microscopy studies of the bone-hydroxylapatite interface. J Biomed Mater Res. 1984;18(7):719-26.

15. Yu H, Wooley PH, Yang SY. Biocompatibility of Poly-epsilon-caprolactone-hydroxyapatite composite on mouse bone marrow-derived osteoblasts and endothelial cells. J Orthop Surg Res. 2009;4:5.

16. Furuzono T, Yasuda S, Kimura T, Kyotani S, Tanaka J, Kishida A. Nano-scaled hydroxyapatite/polymer composite IV. Fabrication and cell adhesion properties of a three-dimensional scaffold made of composite material with a silk fibroin substrate to develop a percutaneous device. J Artif Organs. 2004;7(3):137-44.

17. Midy V, Hollande E, Rey C, Dard M, Plouet J. Adsorption of vascular endothelial growth factor to two different apatitic materials and its release. J Mater Sci Mater Med. 2001;12(4):293-8.

18. Stayton PS, Drobny GP, Shaw WJ, Long JR, Gilbert M. Molecular recognition at the protein-hydroxyapatite interface. Crit Rev Oral Biol Med. 2003;14(5):370-6.

19. Uemura T, Nemoto A, Liu Y-k, Kojima H, Dong J, Yabe T, et al. Osteopontin involvement in bone remodeling and its effects on in vivo osteogenic potential of bone marrow-derived osteoblasts/porous hydroxyapatite constructs. Mater Sci Eng, C. 2001;17(1–2):33-6.

20. Sun H, Mei L, Song C, Cui X, Wang P. The in vivo degradation, absorption and excretion of PCL-based implant. Biomaterials. 2006;27(9):1735-40.

21. Ripamonti U. Soluble, insoluble and geometric signals sculpt the architecture of mineralized tissues. J Cell Mol Med. 2004;8(2):169-80.

22. Kim HW, Knowles JC, Kim HE. Effect of biphasic calcium phosphates on drug release and biological and mechanical properties of poly(epsilon-caprolactone) composite membranes. J Biomed Mater Res A. 2004;70(3):467-79.

23. Schiller C, Epple M. Carbonated calcium phosphates are suitable pH-stabilising fillers for biodegradable polyesters. Biomaterials. 2003;24(12):2037-43.

24. Agrawal CM, Athanasiou KA. Technique to control pH in vicinity of biodegrading PLA-PGA implants. J Biomed Mater Res. 1997;38(2):105-14.

25. Arinzeh TL, Tran T, McAlary J, Daculsi G. A comparative study of biphasic calcium phosphate ceramics for human mesenchymal stem-cell-induced bone formation. Biomaterials. 2005;26(17):3631-8.

26. Lomelino Rde O, Castro S, II, Linhares AB, Alves GG, Santos SR, Gameiro VS, et al. The association of human primary bone cells with biphasic calcium phosphate (betaTCP/HA 70:30) granules increases bone repair. J Mater Sci Mater Med. 2012;23(3):781-8.

27. Nery EB, LeGeros RZ, Lynch KL, Lee K. Tissue response to biphasic calcium phosphate ceramic with different ratios of HA/beta TCP in periodontal osseous defects. J Periodontol. 1992;63(9):729-35.

28. Ebrahimi M, Pripatnanont P, Suttapreyasri S, Monmaturapoj N. In vitro biocompatibility analysis of novel nano-biphasic calcium phosphate scaffolds in different composition ratios. J Biomed Mater Res B Appl Biomater. 2014;102(1):52-61.

29. Ma S, Yang Y, Carnes DL, Kim K, Park S, Oh SH, et al. Effects of dissolved calcium and phosphorous on osteoblast responses. J Oral Implantol. 2005;31(2):61-7.

30. Thuaksuban N, Luntheng T, Monmaturapoj N. Physical characteristics and biocompatibility of the polycaprolactone-biphasic calcium phosphate scaffolds fabricated using the modified melt stretching and multilayer deposition. J Biomater Appl. 2016;30(10):1460-72.

31. Bignon A, Chouteau J, Chevalier J, Fantozzi G, Carret JP, Chavassieux P, et al. Effect of micro- and macroporosity of bone substitutes on their mechanical properties and cellular response. J Mater Sci Mater Med. 2003;14(12):1089-97.

32. Gauthier O, Bouler JM, Aguado E, Pilet P, Daculsi G. Macroporous biphasic calcium phosphate ceramics: influence of macropore diameter and macroporosity percentage on bone ingrowth. Biomaterials. 1998;19(1–3):133-39.

33. LeGeros RZ. Properties of osteoconductive biomaterials: calcium phosphates. Clin Orthop Relat Res. 2002(395):81-98.

34. Lee SH, Shin H. Matrices and scaffolds for delivery of bioactive molecules in bone and cartilage tissue engineering. Adv Drug Deliv Rev. 2007;59(4-5):339-59.

35. Thuaksuban N, Luntheng T, Pannak R, Monmaturapoj N, Boonyaphiphat P. Biocompatibility and degradability of novel polycaprolatone – biphasic calcium phosphate scaffolds used for bone tissue engineering. Int J Oral Maxillofac. 2015;44, Supplement 1:e297.

36. Beck GR, Jr. Inorganic phosphate as a signaling molecule in osteoblast differentiation. J Cell Biochem. 2003;90(2):234-43.

37. Bingham PJ, Raisz LG. Bone growth in organ culture: effects of phosphate and other nutrients on bone and cartilage. Calcif Tissue Res. 1974;14(1):31-48.

38. Godwin SL, Soltoff SP. Extracellular calcium and platelet-derived growth factor promote receptor-mediated chemotaxis in osteoblasts through different signaling pathways. J Biol Chem. 1997;272(17):11307-12.

39. Chai YC, Carlier A, Bolander J, Roberts SJ, Geris L, Schrooten J, et al. Current views on calcium phosphate osteogenicity and the translation into effective bone regeneration strategies. Acta Biomater. 2012;8(11):3876-87.

40. Chou YF, Huang W, Dunn JC, Miller TA, Wu BM. The effect of biomimetic apatite structure on osteoblast viability, proliferation, and gene expression. Biomaterials. 2005;26(3):285-95.

41. Adams CS, Mansfield K, Perlot RL, Shapiro IM. Matrix regulation of skeletal cell apoptosis. Role of calcium and phosphate ions. JBiolChem. 2001;276(23):20316-22.

42. Tsigkou O, Jones JR, Polak JM, Stevens MM. Differentiation of fetal osteoblasts and formation of mineralized bone nodules by 45S5 Bioglass conditioned medium in the absence of osteogenic supplements. Biomaterials. 2009;30(21):3542-50.

43. Villalona GA, Udelsman B, Duncan DR, McGillicuddy E, Sawh-Martinez RF, Hibino N, et al. Cell-seeding techniques in vascular tissue engineering. Tissue Eng Part B Rev. 2010;16(3):341-50.

44. Zhou YF, Sae-Lim V, Chou AM, Hutmacher DW, Lim TM. Does seeding density affect in vitro mineral nodules formation in novel composite scaffolds? J Biomed Mater Res A. 2006;78(1):183-93.

45. Beck GR, Jr., Moran E, Knecht N. Inorganic phosphate regulates multiple genes during osteoblast differentiation, including Nrf2. Exp Cell Res. 2003;288(2):288-300.

46. Maeno S, Niki Y, Matsumoto H, Morioka H, Yatabe T, Funayama A, et al. The effect of calcium ion concentration on osteoblast viability, proliferation and differentiation in monolayer and 3D culture. Biomaterials. 2005;26(23):4847-55.

47. Liu YK, Lu QZ, Pei R, Ji HJ, Zhou GS, Zhao XL, et al. The effect of extracellular calcium and inorganic phosphate on the growth and osteogenic differentiation of mesenchymal stem cells in vitro: implication for bone tissue engineering. Biomed Mater. 2009;4(2):025004.

48. Barrere F, van Blitterswijk CA, de Groot K. Bone regeneration: molecular and cellular interactions with calcium phosphate ceramics. Int J Nanomedicine. 2006;1(3):317-32.

Appendix A :

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

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

Thunmaruk Luntheng[a*], Nuttawut Thuaksuban[a] and Naruporn Monmaturapoj[b]

[a]Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Prince of Songkla University, Hatyai, Songkhla, Thailand, 90112

[b]National Metal and Materials Technology Center, Thailand Science Park, Pathumthani, Thailand, 12120

* Corresponding author. E-mail address: thunmaruk@hotmail.com

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 homogenously 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 ($\overline{M}_n$ 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 immerged 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.

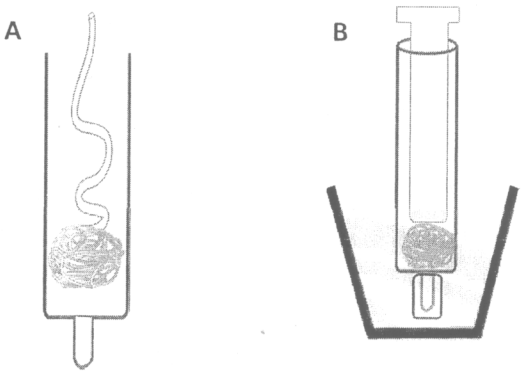


Figure 1 The fabrication process of the PCL-BCP scaffolds using the mMSMD technique; A: the PCL-BCP filament was put into a glass mold and compressed, B: the mold was immersed into warm water allowing the contact points of the filament to fuse together and form a 3-D scaffold.

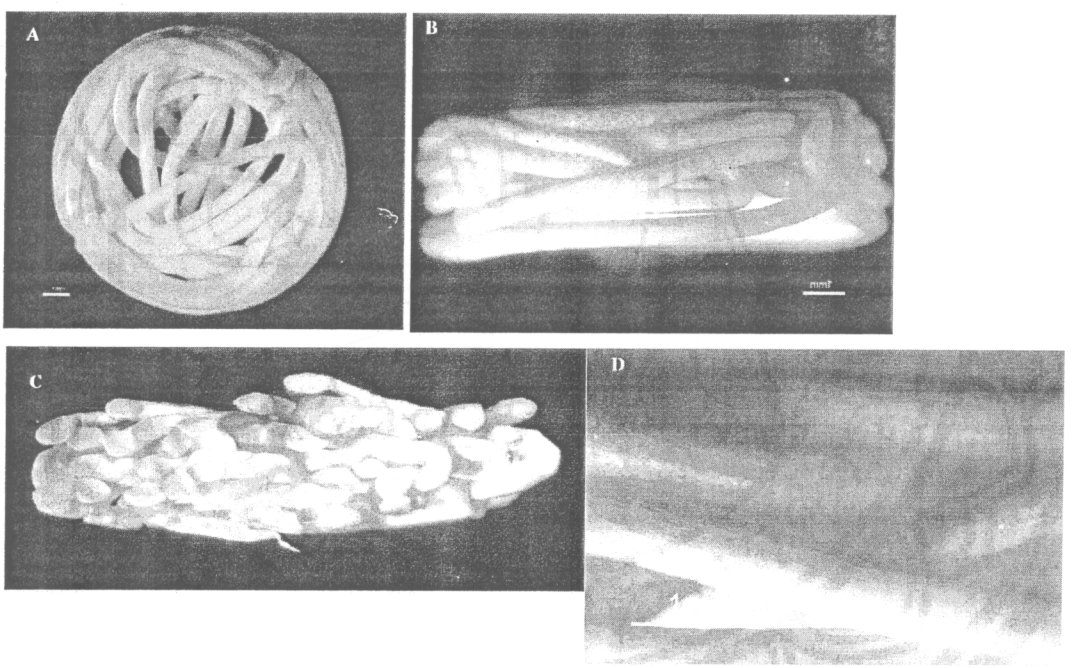


Figure 2 The stereomicroscope images demonstrated the morphologies of the PCL-BCP scaffold specimens; A: superior view, B: lateral view, C: cross-sectional view and D: the magnified picture focuses on the rough surface architecture of the scaffold.

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 μg/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% CO_2 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 10^4 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% CO_2 at 37°C to allow for the cell attachment. After that, the scaffolds of both groups were immerged 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% CO_2 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% CO_2 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 µL 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.

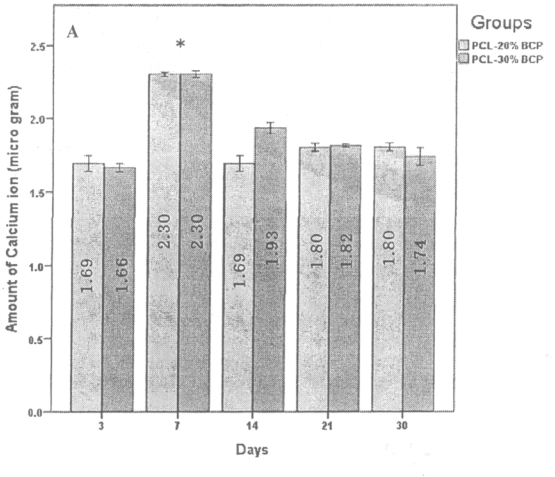
Statistical analysis

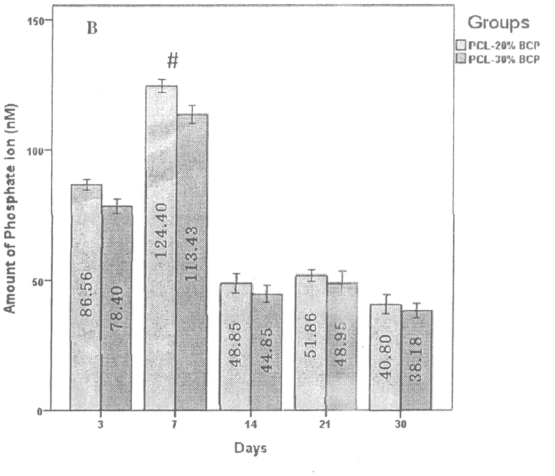
The data was analyzed using statistics analysis software (SPSS, version 14.0, USA). One-way Analysis of Variance (ANOVA) followed by Tukey HSD was applied to compare the differences of the amounts of ions and the parameters of cytocompatibility tests among the experiment groups and the control group. 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 immerged 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 ($p > 0.05$).





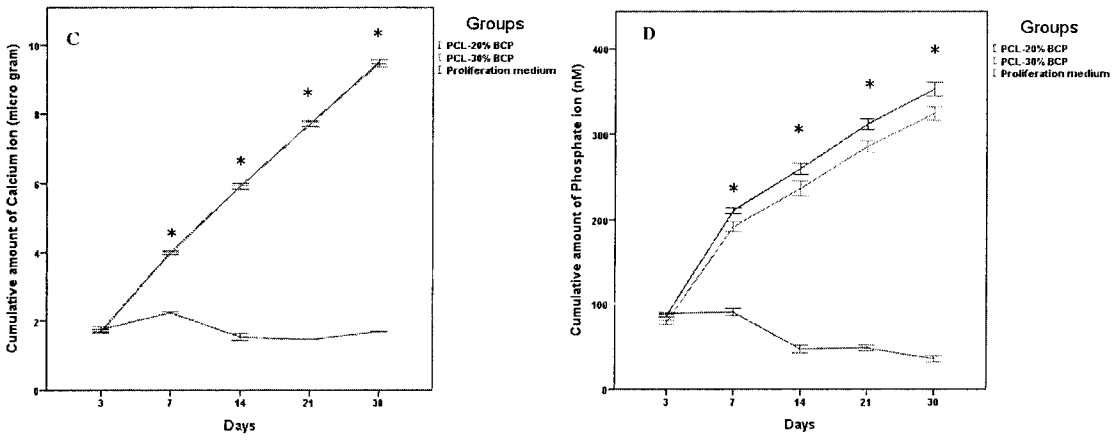


Figure 3 A and B; The graphs demonstrate profiles of calcium and phosphate ions released from the scaffolds over 30 days. The maximum release of both ions were detected on day 7, which was significantly greater than the other days (*, #; ANOVA, $p < 0.05$). The releasing decreased thereafter until reaching 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; $p = 0.00$)

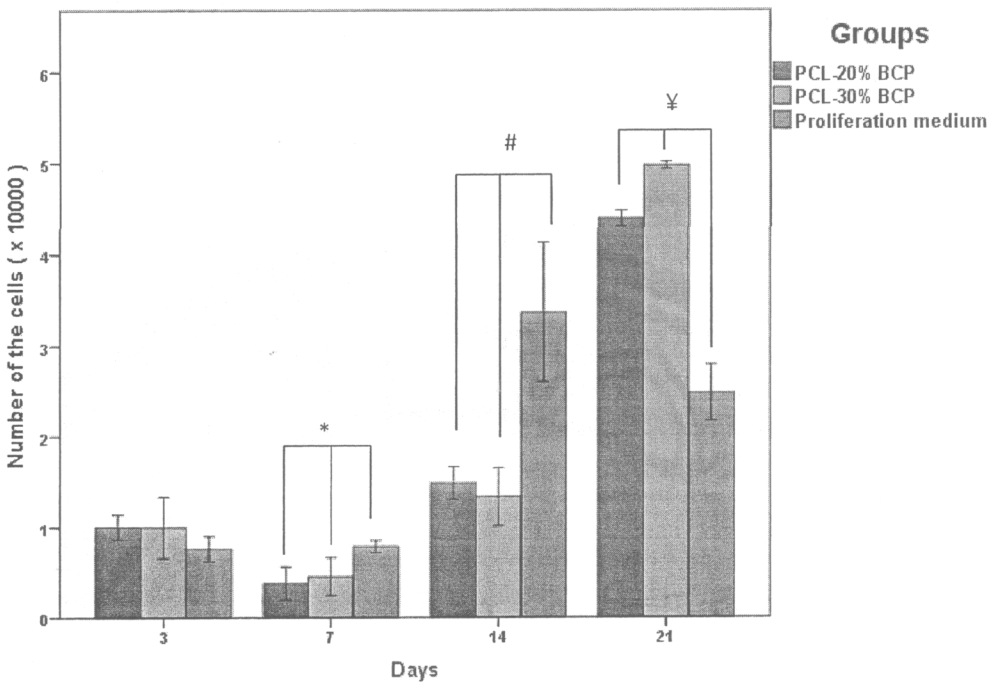


Figure 4 The bar graph shows that amounts of the cells in all groups slightly decreased on day 7, and then remarkably increased on day 14. On day 21, the amounts of the cells in proliferation medium + PCL-20% BCP scaffold and proliferation medium + PCL-30% BCP scaffold continued increased, whilst, those 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.

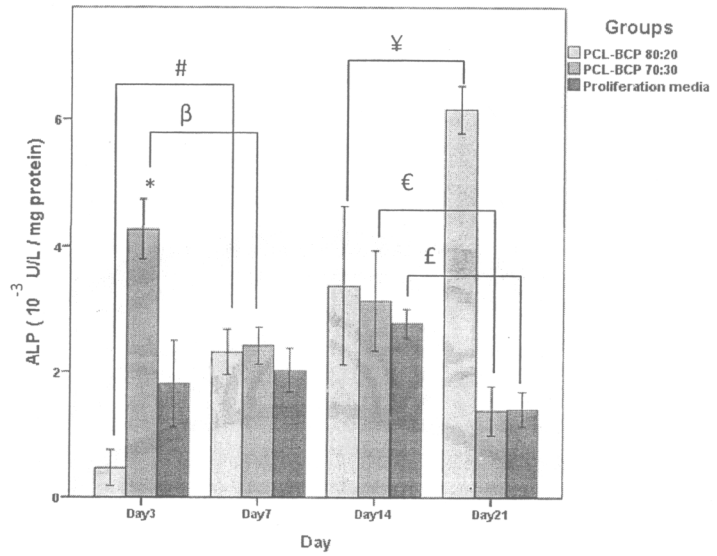


Figure 5 The bar graphs demonstrate ALP activities of the cell-scaffold constructs over 21 days. On day 3, the lowest levels of ALP were detected in proliferation medium + PCL-20% BCP scaffold, whilst, the maximum level was found in proliferation medium + PCL-30% BCP scaffold (significantly greater than 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).

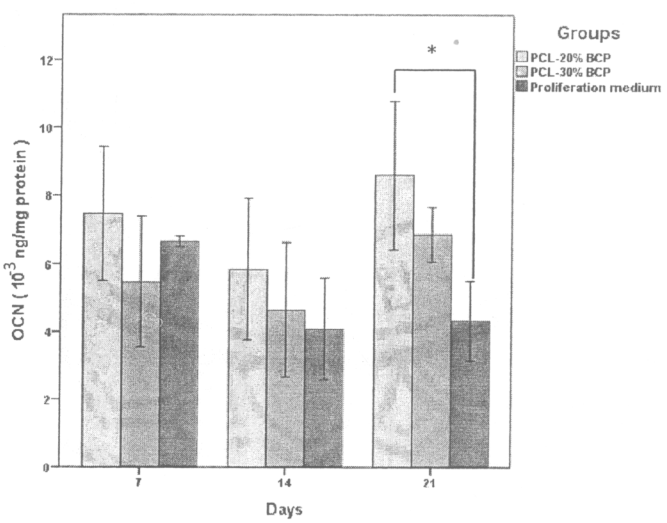


Figure 6 The bar graphs demonstrate the OCN levels of the cell-scaffolds constructs over 21 days. On day 7, the maximum level of OCN was detected in proliferation medium + PCL-20% BCP scaffold followed by the cells in proliferation medium alone and proliferation medium + PCL-30% BCP scaffold. On day 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 form 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 implies 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.

References

Arinzeh, T. L., Tran, T., Mcalary, J., & Daculsi, G. (2005). A comparative study of biphasic calcium phosphate ceramics for human mesenchymal stem-cell-induced bone formation. *Biomaterials, 26*(17), 3631-3638.

Beck, G. R., Jr., Moran, E., & Knecht, N. (2003). Inorganic phosphate regulates multiple genes during osteoblast differentiation, including Nrf2. *Exp Cell Res, 288*(2), 288-300.

Bingham, P. J., & Raisz, L. G. (1974). Bone growth in organ culture: effects of phosphate and other nutrients on bone and cartilage. *Calcif Tissue Res, 14*(1), 31-48.

Dorozhkin, S. V. (2010). Bioceramics of calcium orthophosphates. *Biomaterials, 31*(7), 1465-1485. doi: 10.1016/j.biomaterials.2009.11.050

Ebrahimi, M., Pripatnanont, P., Suttapreyasri, S., & Monmaturapoj, N. (2014). In vitro biocompatibility analysis of novel nano-biphasic calcium phosphate scaffolds in different composition ratios. *J Biomed Mater Res B Appl Biomater, 102*(1), 52-61. doi: 10.1002/jbm.b.32979

Engelberg, I., & Kohn, J. (1991). Physico-mechanical properties of degradable polymers used in medical applications: a comparative study. *Biomaterials, 12*(3), 292-304.

Godwin, S. L., & Soltoff, S. P. (1997). Extracellular calcium and platelet-derived growth factor promote receptor-mediated chemotaxis in osteoblasts through different signaling pathways. *J Biol Chem, 272*(17), 11307-11312.

Lam, C. X., Hutmacher, D. W., Schantz, J. T., Woodruff, M. A., & Teoh, S. H. (2009). Evaluation of polycaprolactone scaffold degradation for 6 months in vitro and in vivo. *J Biomed Mater Res A, 90*(3), 906-919. doi: 10.1002/ jbm.a. 32052

Lei, Y., Rai, B., Ho, K. H., & Teoh, S. H. (2007). In vitro degradation of novel bioactive polycaprolactone—20% tricalcium phosphate composite scaffolds for bone engineering. *Mater Sci Eng C, 27*(2), 293-298. doi: 10.1016/j.msec. 2006.05.006

Lomelino Rde, O., Castro, S., II, Linhares, A. B., Alves, G. G., Santos, S. R., Gameiro, V. S., . . . Granjeiro, J. M. (2012). The association of human primary bone cells with biphasic calcium phosphate (betaTCP/HA 70:30) granules increases bone repair. *J Mater Sci Mater Med, 23*(3), 781-788. doi: 10.1007/s10856-011-4530-1

Ma, S., Yang, Y., Carnes, D. L., Kim, K., Park, S., Oh, S. H., & Ong, J. L. (2005). Effects of dissolved calcium and phosphorous on osteoblast responses. *J Oral Implantol, 31*(2), 61-67. doi: 10.1563/0-742.1

Maeno, S., Niki, Y., Matsumoto, H., Morioka, H., Yatabe, T., Funayama, A., . . . Tanaka, J. (2005). The effect of calcium ion concentration on osteoblast viability, proliferation and differentiation in monolayer and 3D culture. *Biomaterials, 26*(23), 4847-4855. doi: 10.1016/j.biomaterials. 2005.0 1.006

Nery, E. B., LeGeros, R. Z., Lynch, K. L., & Lee, K. (1992). Tissue response to biphasic calcium phosphate ceramic with different ratios of HA/beta TCP in periodontal osseous defects. *J Periodontol, 63*(9), 729-735. doi: 10.1902/jop. 1992.63. 9. 729

Rai, B., Teoh, S. H., & Ho, K. H. (2005). An in vitro evaluation of PCL-TCP composites as delivery systems for platelet-rich plasma. *J Control Release, 107*(2), 330-342. doi: 10.1016/j.jconrel. 2005. 07.002

Salgado, A. J., Coutinho, O. P., & Reis, R. L. (2004). Bone tissue engineering: state of the art and future trends. *Macromol Biosci, 4*(8), 743-765. doi: 10.1002/mabi.200400026

Schantz, J. T., Hutmacher, D. W., Lam, C. X., Brinkmann, M., Wong, K. M., Lim, T. C., . . . Teoh, S. H. (2003). Repair of calvarial defects with customised tissue-engineered bone grafts II. Evaluation of cellular efficiency and efficacy in vivo. *Tissue Eng, 9 Suppl 1*, S127-139. doi: 10. 1089/10763270360697030

Tay, B. Y., Zhang, S. X., Myint, M. H., Ng, F. L., Chandrasekaran, M., & Tan, L. K. A. (2007). Processing of polycaprolactone porous structure for scaffold development. *J Mater Process Technol, 182*(1-3), 117-121. doi: 10.1016/j.jmatprotec. 2006.07.016

Thuaksuban, N., Nuntanaranont, T., Pattanachot, W., Suttapreyasri, S., & Cheung, L. K. (2011). Biodegradable polycaprolactone-chitosan three-dimensional scaffolds fabricated by melt stretching and multilayer deposition for bone tissue engineering: assessment of the physical properties and cellular response. *Biomed Mater, 6*(1), 015009. doi: 10.1088/1748-6041/6/1/015009

Thuaksuban, N., Nuntanaranont, T., Suttapreyasri, S., Pattanachot, W., Sutin, K., & Cheung, L. K. (2013). Biomechanical properties of novel biodegradable poly epsilon-caprolactone-chitosan scaffolds. *J Investig Clin Dent, 4*(1), 26-33. doi: 10.1111/j.2041-1626.2012.00131.x

Appendix B :

Physical characteristics and biocompatibility of the polycaprolactone-biphasic calcium phosphate scaffolds fabricated using the modified melt stretching and multilayer deposition

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Physical characteristics and biocompatibility of the polycaprolactone–biphasic calcium phosphate scaffolds fabricated using the modified melt stretching and multilayer deposition

Nuttawut Thuaksuban[1], Thunmaruk Luntheng[1] and Naruporn Monmaturapoj[2]

Abstract

Physical properties and biocompatibility of polycaprolactone (PCL)–biphasic calcium phosphate (BCP) scaffolds fabricated by the modified melt stretching and multilayer deposition (mMSMD) technique were evaluated in vitro. The PCL–BCP scaffold specimens included group A; PCL: BCP (wt%) = 80:20 and group B; 70:30. Mechanical properties of the scaffolds were assessed using a universal testing machine. Degradation behaviors of the scaffolds were assessed over 60 days. The amount of calcium and phosphate ions released from the scaffolds was detected over 30 days. Attachment and growth of osteoblasts on the scaffolds and indirect cytocompatibility to those cells were evaluated. The results showed that the scaffolds of both groups could withstand compressive forces on their superior aspect very well; however, their lateral aspect could only withstand light forces. Degradation of the scaffolds over 2 months was low (group A = $1.92 \pm 0.47\%$ and group B = $2.9 \pm 1.3\%$, $p > 0.05$). The concentrations of calcium and phosphate ions released from the scaffolds of both groups significantly increased on day 7 ($p < 0.05$). Growth of the cells seemed to relate to accumulative increase in those ions. All results between the two ratios of the scaffolds were not statistically different.

Keywords

Scaffold, biphasic calcium phosphate, hydroxyapatite, tricalcium phosphate, polycarprolactone

Introduction

In the field of Oral and Maxillofacial Surgery, reconstructing large bone defects in craniofacial regions using various grafting techniques is still very challenging. Over the last several years, using synthetic bone substitutes instead of autogenous bone grafts has become more popular. However, up to now, there is no consensus for the best bone substitution due to the fact that each graft material has its own unique properties. Ideally, the bone substitutes should act as scaffolds for bone cell in-growth, survival, and regeneration. They should be biodegradable acting as temporary matrices for vascularization, cell proliferation, and extracellular matrix deposition until the new bone is totally restored. The rate of degradation should be commensurate with bone regeneration, whilst the mechanical strength of the scaffolds should be maintained during this period. In addition, their structures should consist of three-dimensions (3D) with appropriate pore size and interconnecting pore systems for

[1]Faculty of Dentistry, Department of Oral and Maxillofacial Surgery, Prince of Songkla University, Hatyai, Songkhla, Thailand
[2]National Metal and Materials Technology Center, Thailand Science Park, Pathumthani, Thailand

Corresponding author:
Nuttawut Thuaksuban, Department of Oral and Maxillofacial Surgery, Prince of Songkla University, Hatyai, Songkhla 90112, Thailand.
Email: nuttawut.t@psu.ac.th

transporting nutrition and bone cell in-growth throughout the scaffolds.[1] Our research team developed the novel technique of melt stretching and multilayer deposition (MSMD) specifically for fabricating 3D scaffolds for bone regeneration.[2,3] The MSMD scaffolds are specifically designed to be an appropriate interconnecting pore system for enhancing osteogenesis. A microgroove pattern, typically found on their surfaces, proved to support attachment of osteoblasts.[2] In addition, the mechanical properties of the scaffolds are suitable for withstanding forces occurring in real circumstances of reconstruction in the oral and maxillofacial region.[3] Recently, to make the fabricating process easier and more practical, the steps of MSMD were simplified and were thus called, "modified MSMD (mMSMD)", for fabricating the novel poly-ε-caprolactone (PCL)–biphasic calcium phosphate (BCP) 3D scaffolds. PCL and BCP are very popular biomaterials and are widely used for bone tissue engineering, thus the combination of the two materials are targeted for their synergistic properties. PCL has been approved by the Food and Drug Administration (FDA) as a medical and drug delivery device, having extensive support both in vitro and in vivo studies.[4–7] It degrades by a hydrolytic mechanism under physiological conditions and produces a less acidic environment when compared to other polyesters. However, since it possesses hydrophobic properties, PCL normally takes more than 24 months for complete degrading,[8] which is not commensurate with the bone remodeling process. Currently, PCL-based scaffolds are usually combined with some ceramic fillers for enhancing bioactivities and accelerating degradation rates, and some of the products are available on the market. One of them is the PCL scaffold containing 20% β-tricalcium phosphate (β-TCP) fabricated by fused deposition modeling (FDM). These scaffolds have been proven to support proliferation and differentiation of the osteoprogenitor cell and in vivo bone formation.[9–18] The major advantage of the PCL–TCP scaffolds is that a calcium-rich layer forming on their surfaces during dissolution of the TCP serves as a template for hydroxyapatite growth. Thus, osteoblasts can grow and differentiate on that apatite layer to form an extracellular matrix. In addition, it is claimed that the surrounding bone comes in direct contact with the surface of the layer.[19] BCP, another popular ceramic used for that purpose, is a combination of two ceramic phases aiming for controlled bioactivity and balanced biodegradation.[20] BCP consists of the stable phase of hydroxyapatite (HA) and the more soluble phase of β-TCP. In the living tissue, acidic condition initiates partial dissolution of HA causing releasing of Ca^{2+}, HPO_4^{2-}, and PO_4^{3-} and increasing supersaturation with respect to calcium phosphate phase. Consequently, a hydroxyl carbonate apatite (HCA) layer slowly forms and bonds with bone.[21–24] Although various composition ratios have been studied extensively in vitro and in vivo, the optimum ratio of BCP for using as bone substitutes or scaffolds for bone tissue engineering is still obscure. Most of the previous studies fabricated BCP using higher ratios of HA than those of TCP in order to improve mechanical stability of the materials, but some studies supported better results by using higher ratios of TCP.[25–29] Arinzeh et al.[25] performed an in vivo study to determine the optimum ratio of HA and TCP that induced human mesenchymal stem cells (hMSC) and promoted new bone formation. Six types of ceramics including 100% HA, 100% TCP and HA/TCP at the ratios of 76/24, 63/37, 56/44, and 20/80 seeded with hMSC were implanted subcutaneously into the backs of severely immunodeficient genetically disordered mice. The results showed that ceramics containing a high ratio of TCP (HA/TCP = 20/80) induced more hMSC and bone formation than those containing high ratios of HA. Lomelino et al.[29] evaluated the suitability of BCP granule (β-TCP/HA = 70:30) as a potential carrier for cell-guided bone therapy. Calvarial bone defects (5 mm in diameter) in Wistar rats were filled with autogenous bone grafts, the BCP granules and the granules combined with human bone cells. The results showed that levels of new bone formation over 45 days of the BCP granules combined with the cells were similar to those of the autogenous bone, whilst those of the BCP granules alone were less than those two groups; however, no significant difference was detected. Recently, our institute in corporate with The National Metal and Materials Technology Center of Thailand (MTEC) prepared BCP particles as a bone substitute in different compositions of HA/β-TCP with ratios of 30/70, 40/60, and 50/50.[26,27] Proliferation and differentiation of the mouse osteoblast (MC3T3-E1) seeded on those particles were assessed in vitro over 19 days. The results indicated that the cells on BCP 30/70 grew fastest and expressed the highest alkaline phosphatase activity earlier than the other groups. However, on day 19, the highest osteocalcin activity was detected in the 50/50 group followed by the 30/70 and 40/60 group, respectively. Therefore, it implies that BCP containing the higher ratios of TCP at 30/70 would support proliferation and early phases of differentiation of the osteoblast cells, whilst the higher HA ratio (50/50) would support the late phase of differentiation. In this study, BCP 30/70 seemed to be the best ratio for influencing entire phases of the bone formation. However, the limitations of BCP with high ratios of TCP are mainly due to its weak mechanical properties. Tadashi et al.[30] found that the number of micro-cracks found in BCP composite block grafts increased with higher ratios of β-TCP. They concluded that higher ratios of β-TCP

would decrease the mechanical strength of BCP. In our opinion, combining BCP 30/70 with PCL for fabricating biodegradable scaffolds was a good strategy, not only for increasing bioactivity but also for improving the degradation properties of the scaffolds. In addition, when using the BCP as a filler for PCL-based scaffolds, the effects of its poor mechanical properties would be eliminated. Regarding using either the MSMD or mMSMD technique, which utilizes a melt-blending concept, the two materials could be easily mixed due to the fact that BCP is stable when the temperature is in the range of 100–120°C and at which temperature PCL can melt. Therefore, the melted blend of PCL and BCP can be extruded to be a filament and the 3D scaffolds can be built by using those filaments.[2,3] Initially, we hypothesized that the PCL matrix, which supports the BCP fillers, would diminish its brittle property, whilst the bioactivities and degradation behaviors of the PCL scaffolds would be improved by the action of the filler. In this study, the fabrication technique of mMSMD was introduced and in vitro physical characteristics and biocompatibility of the PCL–BCP mMSMD scaffolds were assessed.

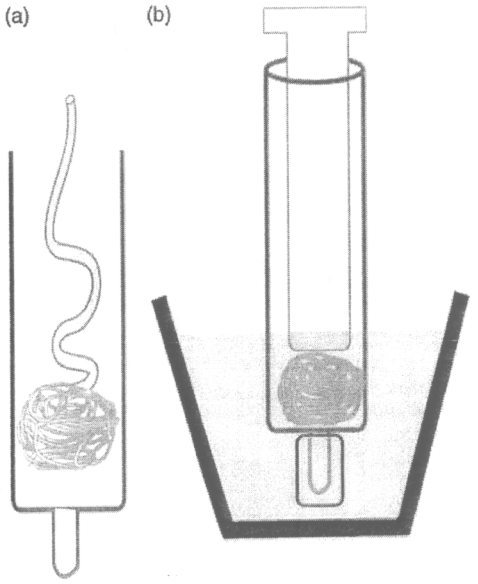

Figure 1. The fabrication process of PCL–BCP scaffolds using the mMSMD technique: (a) the PCL–BCP filament was put into a glass mold and compressed, (b) the mold was immersed into warm water allowing the contact points of the filament to fuse together and form a 3D scaffold.

Materials and methods

Scaffold fabrication and study groups

The study groups were divided into two groups according to the ratios of PCL–BCP: group A = 80:20 ratio and group B = 70:30. 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 microparticles (HA: β-TCP = 30:70, particle size < 75 μm, MTEC, Thailand) were used as the raw materials. The two materials were mixed together with the following ratios of PCL: BCP = 80:20 and 70:30 by weight, and then melted in the melting-extruding machine.[2] 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 diameters and were stocked for fabricating the scaffolds.[2] To fabricate each 3D scaffold (Figure 1), a single filament was cut to be 50 cm in length, put into a 5 cc-glass syringe as a mold, and then compressed by pushing the plunger of the syringe until reaching the reference point of 3 mm above its tip. The tip was sealed by polyvinyl siloxane (3 M ESPE, USA), and then the syringe was immerged into warm double distilled water to allow contacted points of the filaments to fuse together and form a 3D scaffold. The scaffolds were removed from the molds and used as the testing specimens (diameter: 11 mm, height: 3 mm). The specimens used for the cellular experiments were sterilized using ethylene oxide gas.

Physical characteristic evaluations

Scaffold morphologies. Architecture and surface morphology of the scaffolds was examined using a stereomicroscope (Nikon SMZ1500, Japan) and a scanning electron microscope (SEM; JEOL Ltd, Japan) ($n = 3$/group). Micro-computer tomography (μCT; μCT35, SCANCO Medical AG, Switzerland) and its analysis software (μCT 35 Version 4.1) was used to evaluate the porosity of the scaffolds ($n = 3$/group). The porosity (%) was calculated by the following equation

$$\frac{\text{Total volume} - \text{Scaffold volume}}{\text{Total volume}} \times 100$$

Mechanical properties. The scaffolds were immersed in simulated body fluid (SBF) and incubated at 37°C for 24 h before the experiments. SBF prepared in accordance with Kokubo et al.'s study[31] consisted of ion concentrations nearly equal to those of human blood plasma. Compressive forces were applied on the superior aspect and lateral aspect ($n = 5$/group/aspect) of the scaffolds for assessing their compressive strength using a universal testing machine (Lloyd Instrument Ltd., UK). For the superior aspect, each soaked scaffold was horizontally placed on the flat testing platform against the compressing probe (15.77 mm diameter; 5 kN load cell). Vertical force from 0 to 400 N

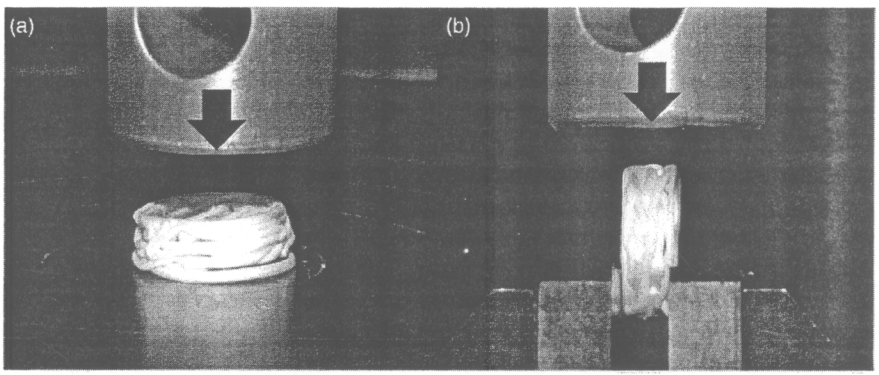

Figure 2. Compression test of the scaffolds: vertical force (arrows) was applied to the superior aspect (a) and lateral aspect (b) of the scaffolds.

was applied to the superior aspect of the scaffold at a cross-head speed of 10 mm/min (Figure 2(a)). For the lateral aspect, each soaked scaffold was vertically immobilized using a custom-made holder, then the vertical force was applied until reaching the limited maximum strain at 30% (Figure 2(b)). The stress–strain behaviors were assessed by load–displacement curves using data analysis software (Nexygen, UK).

Degradability. The scaffolds of each group were immersed in SBF over 60 days for assessment of their degradation in terms of weight loss (%). Each scaffold was weighed (Wd0) using an analytical balance (Satorius, Germany) before immersing in 2 mL of SBF/ well in 24-well tissue culture plates (Nunc, Denmark). The plates were incubated at a constant temperature of 37°C. The scaffolds were collected on day 15, 30, 45, and 60 for measuring their weight loss ($n = 5$/group/time point). At each time point, each scaffold was blotted with filter paper. After being freeze-dried in freeze drying and vacuum concentration equipment (LaboGene ApS, Germany) for 24 h, its dried weight (Wdt) was measured. Weight loss of the scaffold was calculated using the following equation

$$\%\text{Weight loss} = 100 \times (\text{Wd0} - \text{Wdt})/\text{Wd0}$$

Release of calcium and phosphate ions. The scaffolds of each group were left in distilled water (dH_2O) ($n = 25$/group) and in culture medium (alpha-minimum essential medium (α-MEM) (Gibco, Invitrogen, USA) containing 10% fetal bovine serum (FBS) (Gibco, Invitrogen, USA), 10,000 units/mL penicillin/streptomycin (Gibco, Invitrogen, USA), and 250 µg/mL fungizone (Gibco, Invitrogen, USA)) ($n = 25$/group) for detecting release of calcium and phosphate ions over 30 days. The scaffolds were immersed in 2 mL of each solution per well in the tissue culture plates, and then the plates were incubated at a constant temperature of 37°C. On day 3, 7, 14, 21, and 30 thereafter, the scaffolds were moved into next wells and the fresh solutions were 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/well of the sample solutions and mixed gently in a 96-well plate (Nunc, Denmark). 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/well of the sample solutions were placed in a 96-well plate. Thirty microliters of phosphate reagent were added into each well and mixed gently. The plate was incubated at room temperature for 30 min. The absorbance of malachite green and ammonium molybdate formed a chromogenic complex with phosphate ions, which was measured at 700 nm. The levels of OD were compared with a standard curve to calculate the concentration 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 culture medium. The cells were cultivated in 5% CO_2 at 37°C until reaching confluence and then subculturing was conducted. The cells between passages 3 and 6 were used for the experiments.

Morphologies of cell attachment and proliferation. On days 21, 14, 7, and 3 before the experiment, the cells were seeded on the scaffolds of each group ($n = 2$/group/time point) according to the following protocol. Prior to the

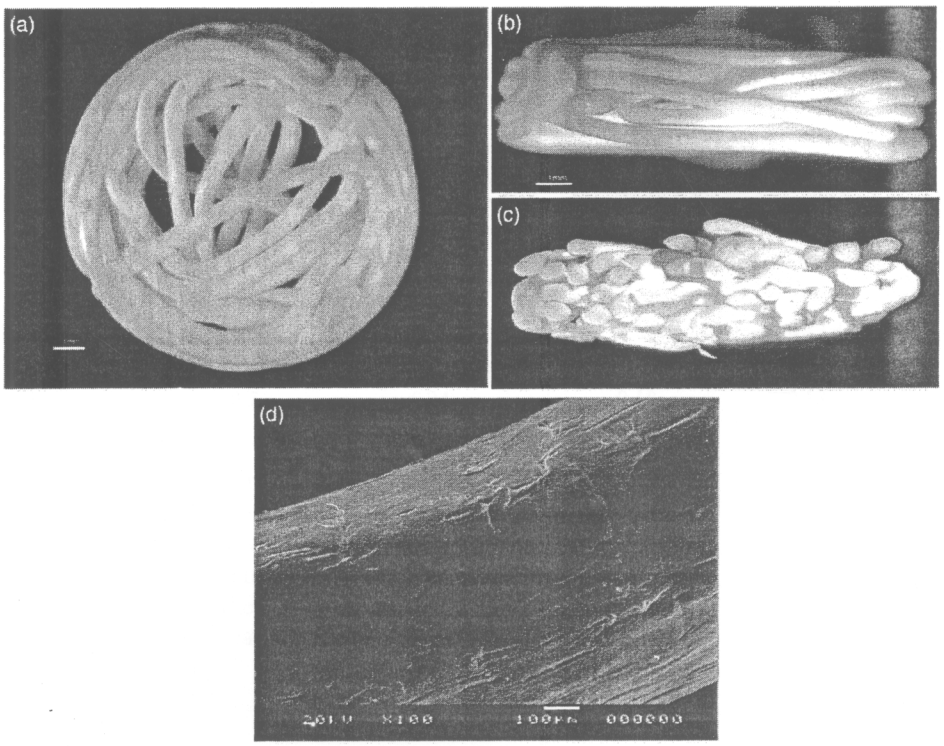

Figure 3. The macro- and micro-structures of the scaffold. The stereomicroscope pictures show that the scaffold had a large and irregular interconnecting pore system: (a) superior aspect, (b) lateral aspect, (c) cross-sectional aspect, (d) The SEM picture shows that the surface of the scaffold had the pattern of irregular microgrooves.

cell seeding, the scaffolds were immersed in the culture medium for 24 h, after that cell-scaffold constructs were made by seeding 1×10^5 cells/scaffold. The constructs were cultivated in 5% CO_2 at 37°C until the experiment. On the day of experiment, the constructs were rinsed with phosphate buffer saline (PBS), and then fixed in 2.5% glutaraldehyde in PBS for 2 h. Afterwards, they were dehydrated in ethanol series of 60–100%, then dried and coated with gold-palladium. Characteristics of the cells on the scaffolds were examined by SEM.

Cytocompatibility test. The experiment was performed in order to evaluate an influence of the releasing ions from the scaffolds on growth of the osteoblast cells. On days 21, 14, 7, and 3 before the experiment, 1×10^4 of the cells were seeded into each well of the 48-well culture plates (Nunc, Denmark) and then 200 µL of the culture medium were added. The plates were left for 3 h in 5% CO_2 at 37°C to allow for cell attachment. After that, the scaffolds of both groups were immerged into each well and secured as close to the bottom of the well as possible ($n = 5$/ group/ time point). The medium without the scaffold was reserved as the control group ($n = 5$/time point). The plates were cultivated in 5% CO_2 at 37°C and the medium was changed every 3 days until the experiment. On the day of experiment, the cell proliferative reagent (WST-1, Roche, Germany) was used to measure activity of mitochondrial dehydrogenases for reflecting the number of viable cells as per the following protocol. For each well, the scaffold and the medium were removed and replaced with 200 µL of the fresh culture medium without FBS and 20 µL of WST-1 solution. The plates were incubated for 4 h in 5% CO_2 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.

Statistical analysis. The data was analyzed using statistics analysis software (SPSS, version 14.0, USA). Macroscopic features of the scaffolds and morphologies of the cells in the cell-scaffold constructs were assessed descriptively. Paired *t*-test was applied to compare the differences of the mechanical parameters between the two groups. One-way analysis of variance (ANOVA) followed by Tukey HSD was applied to compare the differences of the percent weight loss, amount of

Table 1. Summary of the mechanical properties of the scaffolds. For the superior aspect, compressive strength and the Young's modulus of group A were slightly less than those of group B, but no significant difference was found. Regarding the strain at maximum load, the scaffolds of both groups had high elasticity and there was no significant difference found. For the lateral aspect, the data of the maximum load and the compressive strength show that the lateral aspects of the scaffolds of both groups could withstand the minimal forces. The Young's modulus of group A was significantly less than group B (* = significant difference).

	Superior aspect			Lateral aspect		
Groups	Compressive strength (MPa)	Strain at maximum load (%)	Young's modulus (MPa)	Maximum load (N)	Compressive strength (MPa)	Young's modulus (MPa)
A	3.61 ± 0.10	49.40 ± 3.46	15.38 ± 0.46	12.29 ± 3.42	0.12 ± 0.04	1.09 ± 0.48
B	3.83 ± 0.19	50.31 ± 4.70	17.72 ± 3.61	21.56 ± 9.47	0.21 ± 0.10	2.74 ± 1.00
p-Value	0.95	0.76	0.24	0.10	0.13	0.02*

calcium and phosphate ions, and numbers of cells for the time intervals within each group. Dunnett's T3 test was performed when equal variances were not assumed. The paired *t*-test was applied to compare the differences of those parameters between the two groups at each time point. The level of statistical significance was set at a $p < 0.05$.

Results

The physical characteristics

Scaffold morphologies. The architectures and the surface morphologies of the scaffolds were demonstrated in Figure 3. The average porosity of the scaffolds measured by μCT was 73.19 ± 3.31%.

Mechanical properties. The mechanical properties of the scaffolds were demonstrated in Table 1. The data showed that the scaffolds of both groups could withstand the vertical compressive force in the superior direction up to 400 N. The scaffolds could be compressed, and then they could recover to their initial dimensions without distortion. The compressive strength and Young's modulus of both groups were not significantly different. Their strain at maximum load was high and no significant difference between the groups was found. In contrast, the scaffolds of both groups could not withstand the compressive force in the lateral direction. The maximum load and compressive strength of the lateral aspects of the scaffolds were low. The scaffolds collapsed when reaching a strain of 30%.

Degradability. The degradation of the scaffolds was demonstrated in Figure 4. Over 60 days, the scaffolds of both groups slightly degraded with time and no significant difference between the groups was found at each time point (paired *t*-test, $p > 0.05$). On day 60, the maximum percentages of weight loss in group A and group B = 1.92 ± 0.47 and 2.9 ± 1.30, respectively.

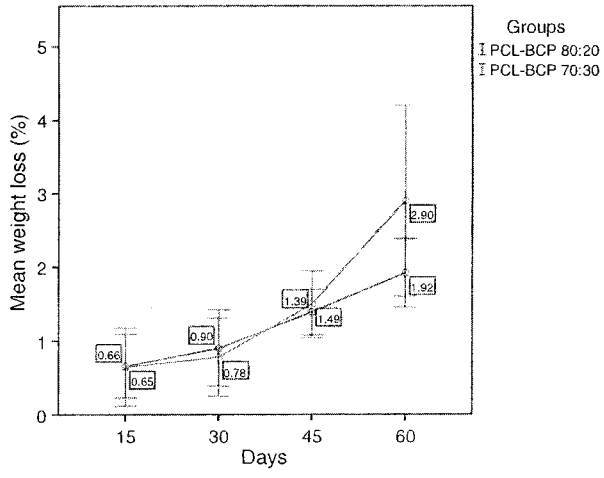

Figure 4. The graph demonstrated that the scaffolds of group A gradually degraded over 60 days, whilst the degradation rate of the scaffolds of group B increased after 45 days. However, the total weight loss of both groups was low.

Release of calcium and phosphate ions. The concentrations of calcium and phosphate ions released from the scaffolds were demonstrated in Figure 5 and 6. The results demonstrated that the scaffolds of both groups could release calcium and phosphate ions over the observation periods when immerged in both dH_2O and the culture medium. However, there was no significant difference between the two groups at each time point (*t*-test, $p > 0.05$). The releasing concentrations of those ions in both groups on day 7 significantly increased more than other days (ANOVA, $p < 0.05$), and then decreased thereafter. In addition, their concentrations in the culture medium were greater than in dH_2O at every time point, except those of group A on day 7. However, no significant difference was found (*t*-test, $p > 0.05$). The cumulative data demonstrated that the accumulation of calcium and phosphate ions of both groups increased with time in both solutions. It was

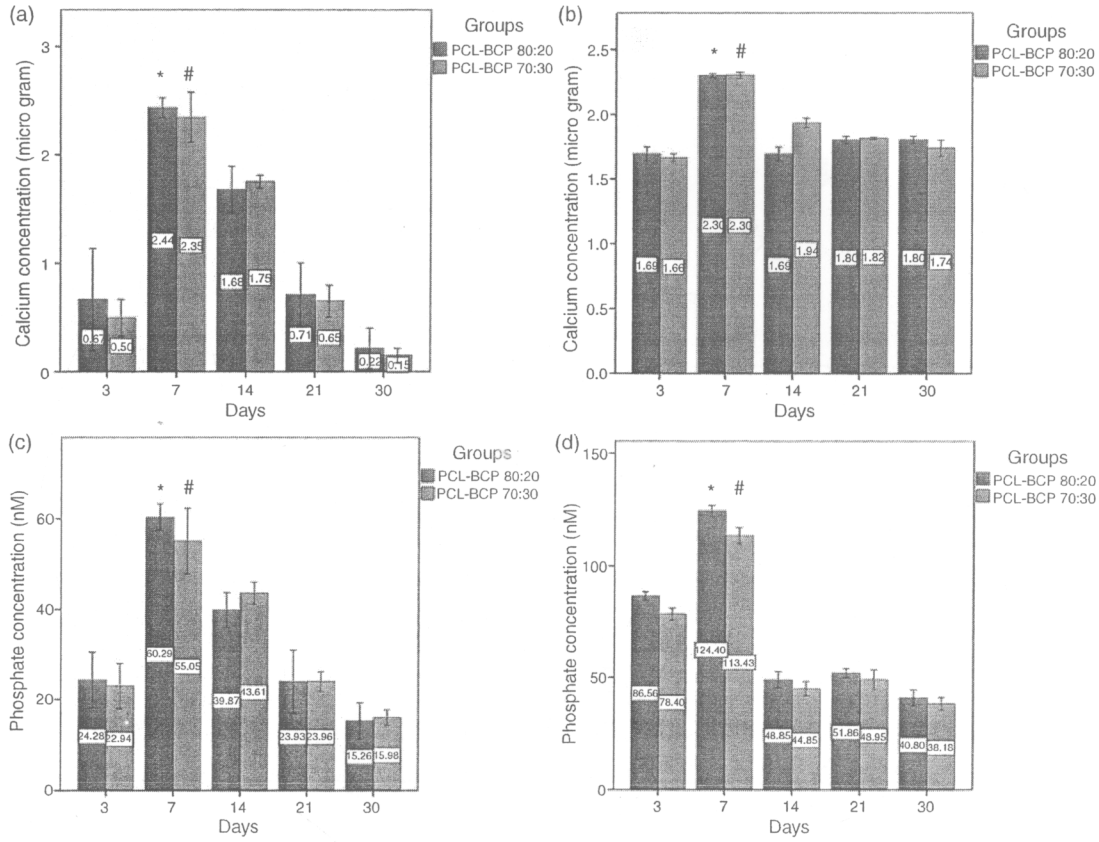

Figure 5. The graphs demonstrate the profiles of releasing calcium and phosphate ions in dH_2O (a, c) and the culture medium (b, d) over 30 days. The maximum release of both ions were detected on day 7, which was significantly greater than any other day (ANOVA: group A, $* = p < 0.05$ and group B, $\# = p < 0.05$), and then the releasing gradually decreased thereafter.

found that the releasing rates of both ions in dH_2O remarkably increased in the first 14 days and then gradually decreased thereafter. In contrast, those rates in the culture medium continued increasing with time after the first 14 days. In addition, the concentrations of phosphate ions in group A were significantly higher than those of group B at all time-points.

Biocompatibility in vitro

Morphologies of cell attachment and proliferation. The SEM pictures demonstrated that the osteoblast cells could attach and grow well throughout the surfaces of the scaffolds (Figure 7). Since day 3 after seeding, it was found that the cells grew in multilayers and formed dense cell-sheets covering the surfaces.

Cytocompatibility test. The amounts of viable cells in both groups at all observation time points were demonstrated in Figure 8. The amounts of the cells of both groups slightly decreased on day 7, and then increased thereafter to reach the maximum growth on day 21. In the control group, the maximum growth of the cells was detected on day 14, and then it decreased on day 21. It was found that the amounts of the cells in the control group on day 7 and 14 were significantly greater than those of group A and B (ANOVA, $p < 0.05$). In contrast, on day 21 those of group A and B were significantly greater than that of the control group (ANOVA, $p = 0.00$).

Discussion

This study introduced a new technique for fabricating polymer–ceramic composite scaffolds. By simplifying the MSMD technique, the mMSMD technique is easier to process allowing surgeons to instantly build 3D scaffolds on the chair side of surgical operations. Therefore, the processing time is remarkably reduced. In addition, fabricating the scaffold within the glass mold is a closed environment preventing contamination during the processing. However, similar to the MSMD technique, the maximum amounts of the BCP filler cannot exceed 30% due to possible fractures of the filaments.[2] Therefore, in this study, the ratios of PCL–BCP at 70:30 and 80:20 were selected for the

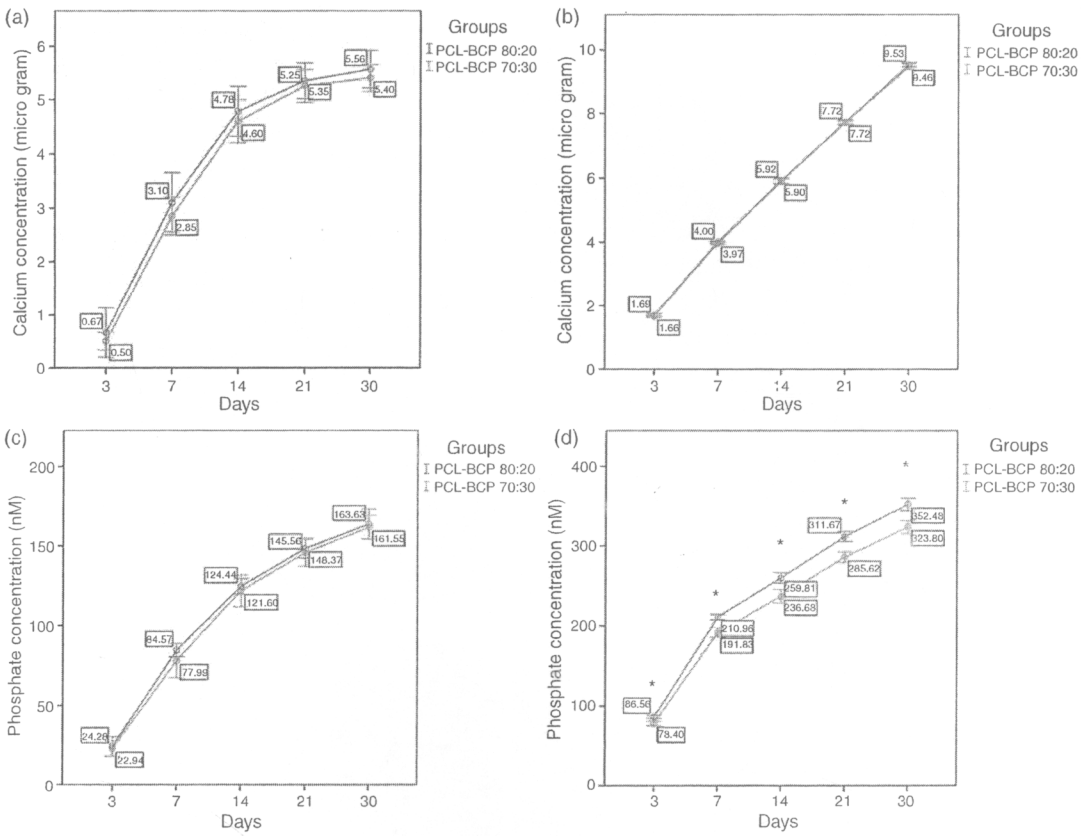

Figure 6. The graphs demonstrate cumulative release of calcium and phosphate ions in dH_2O (a and c) and the culture medium (b and d) over 30 days. The data shows that accumulation of the releasing ions increased with time in both solutions. It was noted that in the culture medium, the concentrations of phosphate ions in group A were significantly higher than those of group B at each time point ($* = p < 0.05$).

experiments for evaluating activities of the BCP filler. Regarding the results of this study, the PCL–BCP mMSMD scaffolds had acceptable physical properties and good biocompatibility for use as the bone substitute. The architecture of the scaffolds consisted of a large interconnecting pore system with high porosity, which was considered to be appropriate for new bone regeneration.[32–35] The mechanical properties of the mMSMD scaffolds were similar to those of the MSMD scaffolds as previously reported.[3] The PCL–BCP mMSMD scaffolds had high elasticity and they could tolerate compressive forces in the superior directions equal to the normal bite force. Nevertheless, the lateral aspects of the scaffolds is still the weak point that can only tolerate minimal compressive forces, therefore, any lateral excessive forces in real grafting situations should be avoided. The profiles of the mechanical properties of PCL–BCP mMSMD scaffolds, human bone, and the PCL MSMD scaffolds are comparatively demonstrated in Table 2. The data shows that the compressive strength of the mMSMD scaffolds is comparable to that of human cancellous bone. In addition, their properties are similar to those of the PCL MSMD scaffolds and there is no statistical difference between the ratios of PCL: BCP = 80:20 and 70:30. Therefore, it implies that the addition of the BCP filler does not compromise that property of the PCL-based scaffolds.

Regarding the degradation test, the PCL–BCP mMSMD scaffolds had a slow degradation rate similar to the PCL–MSMD scaffolds;[2] therefore, the result was against the initial hypothesis due to the fact that the BCP filler did not accelerate the degradation rate of the PCL-based scaffolds. Two possible reasons could be that, firstly, the proportions of the BCP filler were not enough for accelerating the structural degradation of the PCL matrix and secondly, the composition of nonresorbable HA in the BCP might retard the degradation process of the scaffolds. However, in our opinion, the slow degradation property of the PCL–BCP scaffolds is still an advantage because the strength of the scaffolds can be maintained as supportive structures over the entire periods of complete bone remodeling which usually takes around 6 months. In addition, the slow degradation rate of those scaffolds would sustain longer releasing

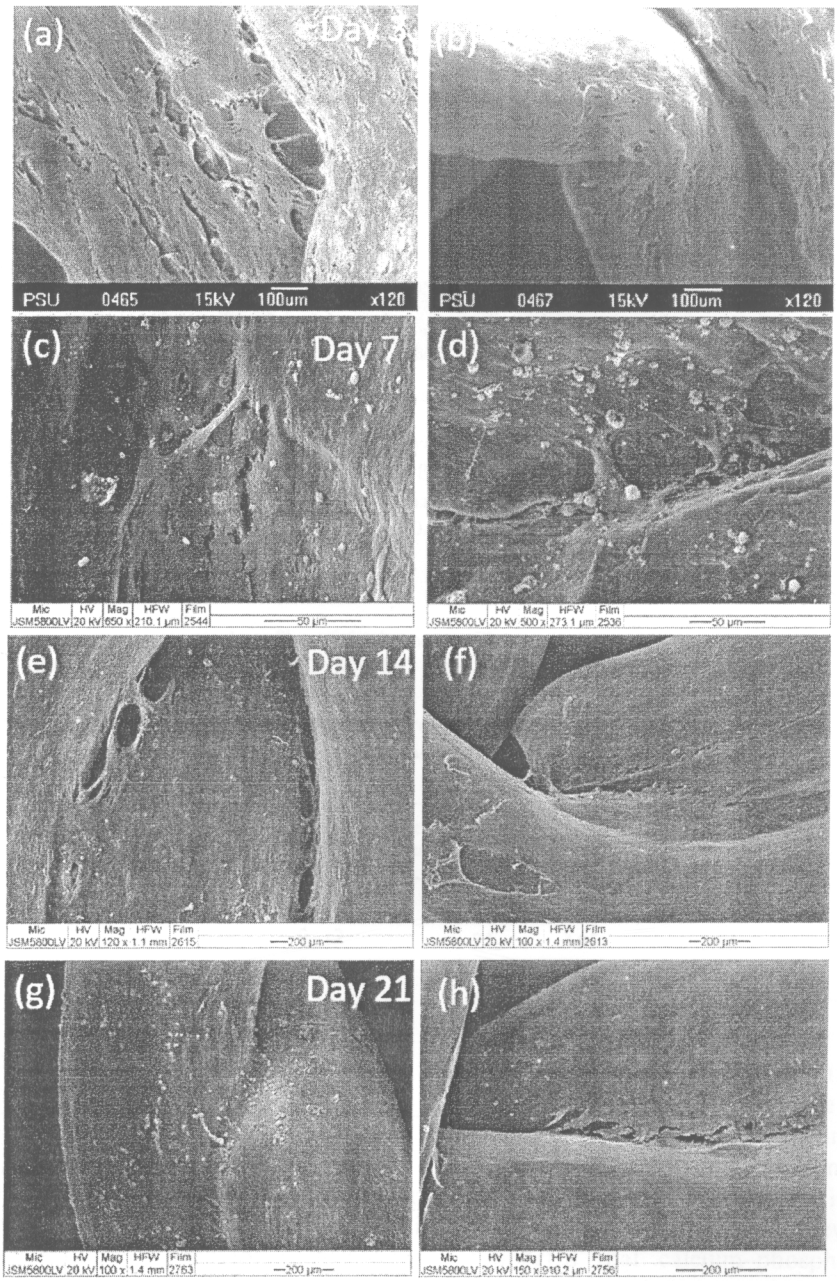

Figure 7. The SEM pictures of the cell-scaffold constructs: (a), (c), (e), (g) group A, and (b), (d), (f), (h) group B. Since day 3, the cells formed dense multilayer cell-sheets covering entire surfaces of the scaffolds and this feature increased with time.

of calcium and phosphate ions, which are essential supports for bone regeneration.

Regarding the previous studies, the degradability of the PCL–ceramic scaffolds were intensively investigated.[17–19] The shortest period of 28 days was to observe the degradation behaviors of the PCL-20%TCP scaffolds in vitro in terms of their weight loss and forming apatite layer on the surfaces when immerged in simulated body fluid.[19] The longer periods were observed in vitro and in rabbit models when those scaffolds were immerged in culture medium or implanted in the calvarial defects.[17,18] It is found that the results of those in vitro studies and our study are corresponding. Within the first 8 weeks (60 days), the percentages of weight loss of the scaffolds were very low (less than 5%).[17,19] However, their degradation rates are remarkably increased (higher than 30% of volume loss) in the living tissue[18] due to the fact that the cellular responses

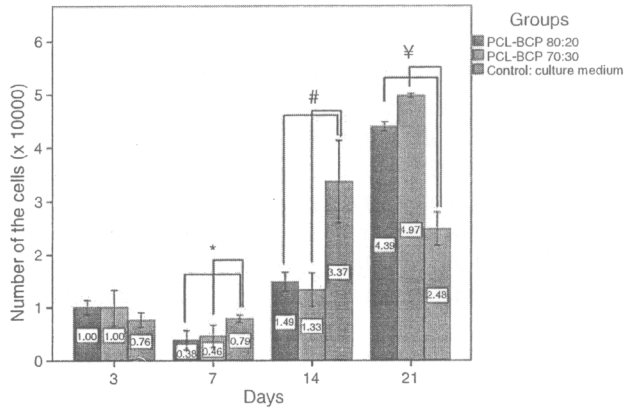

Figure 8. The bar graph shows that the amounts of cells in group A and B slightly decreased on day 7, and then remarkably increased on day 14. On day 21, the amounts of the cells in group A and B continued to increase, whilst those of the control group decreased. (* and # show that the control group was significantly greater than group A and B, $p = 0.00$ and 0.01 respectively, and ¥ shows that the control group was significantly less than group A and B, $p = 0.00$).

Table 2. The table shows the mechanical profiles of the human bone,[31] the PCL MSMD scaffolds and the PCL–BCP mMSMD scaffolds.[2,3] The compressive strength of PCL–BCP mMSMD scaffolds was comparable to human cancellous bone and there was no statistical difference of the mechanical properties among the three types of scaffolds.

	Young's modulus (MPa)	Compressive strength (MPa)
Cortical bone	7000–30,000	100–200
Cancellous bone	50–500	2–12
PCL MSMD scaffolds	13.05 ± 0.7	2.78 ± 0.04
PCL–BCP mMSMD scaffolds 80:20	15.38 ± 0.46	3.61 ± 0.1
PCL–BCP mMSMD scaffolds 70:30	17.72 ± 3.61	3.83 ± 0.19

Table 3. The table shows the average accumulative concentrations (mM) of calcium and phosphate ions releasing from the scaffolds of both groups in the culture medium.

	Calcium ion (mM)		Phosphate ion (mM)	
Day	Group A	Group B	Group A	Group B
3	0.84	0.83	0.087	0.078
7	1.99	1.98	0.211	0.192
14	2.95	2.94	0.259	0.237
21	3.85	3.85	0.312	0.286
30	4.75	4.72	0.352	0.324

are more dominant. Therefore, the extending experiment should be performed in the animal models in order to elucidate degradation behaviors as well as biocompatibility of the PCL–BCP mMSMD scaffolds in the real circumstances of bone reconstruction.

This study revealed that the PCL–BCP scaffolds could maintain releasing calcium and phosphate ions throughout the 30 days. Although the maximum releasing of the ions was detected on day 7 and the rates of releasing decreased thereafter, the amounts of those ions still cumulatively increased with time. Theoretically, the more the BCP amount in the composite, the higher the calcium and phosphate could be released. Unexpectedly, the results showed that the accumulation of those ions of the PCL-20%BCP scaffolds was greater than that of the PCL-30%BCP scaffolds. It could be described that the saturated solution would slow down the release of the ions and it affected the releasing phosphate ion more than the calcium ions particularly in the culture medium. Therefore, it might imply that increasing the amount of BCP filler up to 30% as a limitation would not increase the release of calcium and phosphate ions. Several previous studies supported that calcium–phosphate biomaterials are considered to have osteoinductive property mainly relating with the release of calcium and phosphate ions.[36–40] The effect of those ions are essential for the functions of osteoblast cells and subsequent new bone formation. The calcium ion has potent signals for migration and maturation of pre-osteoblasts required for the bone remodeling process.[41–43] Moreover, this ion induces expression of osteoinductive growth factors including BMP-2 and BMP-4.[44] The phosphate ion is identified as an important signaling molecule that regulates proliferation rate of the cells and bone matrix mineralization. However, some studies demonstrated that those effects were concentration dependence.[45–51] Maeno et al.[45] investigated the effect of various

concentrations of calcium ions on functions of cultured mouse primary osteoblasts. The results showed that low concentration of calcium ions of 2–4 mM and 6–8 mM provided suitable conditions for proliferation and differentiation of the cells respectively. Godwin and Soltoff[46] reported a relation between extracellular calcium concentrations and chemotaxis of MC3T3-E1 osteoblast cell lines. They found that the rates of chemotaxis of the cells correlated with an increase of the calcium concentration within the range of 1.8–5 mM. The other studies reported the maximal effects of calcium ions were in the range of 3–10 mM[47] and 2–8 mM.[48,49] Beck et al.[50] reported that inorganic phosphate is a signaling molecule for altering gene expression of osteoblast cells during their differentiation. By using microarray analysis of phosphate-treated MC3T3-E1 osteoblast cells, they identified that some multiple genes such as Nrf2, which associated with the osteoblast differentiation, were upregulated by an increase in the concentration of phosphate ions. Bingham and Raisz[51] examined the effect of increasing phosphate ions in the range of 1.5–4.5 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. However, addition of high concentration of phosphate ranging from 5 to 7 mM was reported to induce in vitro osteoblast apoptosis and non-physiological mineral deposition.[52] The optimal concentrations of calcium and phosphate ions may vary according to the cellular stages of proliferation and differentiation and there is no optimal concentration that universally stimulate the cells toward successful osteogenesis.[53] Regarding our study, the accumulative concentrations of calcium and phosphate ions in the culture medium over the observation periods were demonstrated in the Table 3. The data shows that the concentrations of calcium ion in the culture medium since day 14 are in the ranges of a suitable environment for supporting the functions of the osteoblasts, whilst those of the phosphate ion seem to be less than the optimum levels. However, the responses of the osteoblast cells would relate to the releasing profiles of those ions. The burst releasing ions on day 7 seemed to produce an inappropriate environment for the growth of the cells; however, a decrease of their releasing rate thereafter would promote proliferation of the cells. For the clinical relevance, the effects of those ions on the differentiation of the bone forming cells directly attaching to the surfaces of the scaffolds should be intensively evaluated both in vitro and in animal models.

This study proved that the PCL–BCP mMSMD scaffolds could act as not only an osteoconductive framework for new bone regeneration, but also they had osteoinductive properties due to their ability to release calcium and phosphate ions that supporting the growth of osteoblasts. In addition, the physical characteristics and the cellular responses of the PCL–BCP scaffolds were not different between using the ratios of 80:20 and 70:30. Therefore, regarding the economical and practical aspects in terms of saving the filler material and being an easier fabricating process, the ratio of PCL: BCP at 80: 20 is considered to be more suitable rather than 70:30.

Conclusion

The technique of mMSMD is very practical for fabricating the PCL–BCP 3D scaffolds. Its major advantages include chair-side fabrication and preventing contamination. BCP is suitable to be a good filler in PCL-based scaffolds and the ratio of PCL: BCP at 80:20 is appropriate for bulk processing. The scaffolds have proven to be biocompatible in vitro and their physical properties would be suitable for intra-oral bone reconstructions. Calcium and phosphate ions, which are essential for new bone formation, can sustain releasing over the period of normal bone formation. In addition, the releasing ions seem to relate to proliferation of the osteoblast cells.

Declaration of Conflicting Interests

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References

1. Salgado AJ, Coutinho OP and Reis RL. Bone tissue engineering: State of the art and future trends. *Macromol Biosci* 2004; 4: 743–765.
2. Thuaksuban N, Nuntanaranont T, Pattanachot W, et al. Biodegradable polycaprolactone-chitosan three-dimensional scaffolds fabricated by melt stretching and multilayer deposition for bone tissue engineering: Assessment of the physical properties and cellular response. *Biomed Mater* 2011; 6: 015009.
3. Thuaksuban N, Nuntanaranont T, Suttapreyasri S, et al. Biomechanical properties of novel biodegradable poly epsilon-caprolactone-chitosan scaffolds. *J Investig Clin Dent* 2013; 4: 26–33.
4. Hutmacher DW. Scaffolds in tissue engineering bone and cartilage. *Biomaterials* 2000; 21: 2529–2543.
5. Tay BY, Zhang SX, Myint MH, et al. Processing of polycaprolactone porous structure for scaffold development. *J Mater Process Technol* 2007; 182: 117–121.

6. Woodruff MA and Hutmacher DW. The return of a forgotten polymer–Polycaprolactone in the 21st century. *Prog Polym Sci* 2010; 35: 1217–1256.
7. Zein I, Hutmacher DW, Tan KC, et al. Fused deposition modeling of novel scaffold architectures for tissue engineering applications. *Biomaterials* 2002; 23: 1169–1185.
8. Sun H, Mei L, Song C, et al. The in vivo degradation, absorption and excretion of PCL-based implant. *Biomaterials* 2006; 27: 1735–1740.
9. Chim H, Hutmacher DW, Chou AM, et al. A comparative analysis of scaffold material modifications for load-bearing applications in bone tissue engineering. *Int J Oral Maxillofac Surg* 2006; 35: 928–934.
10. Rai B, Teoh SH and Ho KH. An in vitro evaluation of PCL-TCP composites as delivery systems for platelet-rich plasma. *J Control Release* 2005; 107: 330–342.
11. Rai B, Lin JL, Lim ZX, et al. Differences between in vitro viability and differentiation and in vivo bone-forming efficacy of human mesenchymal stem cells cultured on PCL-TCP scaffolds. *Biomaterials* 2010; 31: 7960–7970.
12. Sawyer AA, Song SJ, Susanto E, et al. The stimulation of healing within a rat calvarial defect by mPCL-TCP/collagen scaffolds loaded with rhBMP-2. *Biomaterials* 2009; 30: 2479–2488.
13. Schantz JT, Teoh SH, Lim TC, et al. Repair of calvarial defects with customized tissue-engineered bone grafts I. Evaluation of osteogenesis in a three-dimensional culture system. *Tissue Eng* 2003; 9: S113–126.
14. Schantz JT, Hutmacher DW, Lam CX, et al. Repair of calvarial defects with customised tissue-engineered bone grafts II. Evaluation of cellular efficiency and efficacy in vivo. *Tissue Eng* 2003; 9: S127–139.
15. Shao XX, Hutmacher DW, Ho ST, et al. Evaluation of a hybrid scaffold/cell construct in repair of high-load-bearing osteochondral defects in rabbits. *Biomaterials* 2006; 27: 1071–1080.
16. Yefang Z, Hutmacher DW, Varawan SL, et al. Comparison of human alveolar osteoblasts cultured on polymer-ceramic composite scaffolds and tissue culture plates. *Int J Oral Maxillofac Surg* 2007; 36: 137–145.
17. Yeo A, Rai B, Sju E, et al. The degradation profile of novel, bioresorbable PCL-TCP scaffolds: An in vitro and in vivo study. *J Biomed Mater Res A* 2008; 84: 208–218.
18. Yeo A, Wong WJ and Teoh SH. Surface modification of PCL-TCP scaffolds in rabbit calvaria defects: Evaluation of scaffold degradation profile, biomechanical properties and bone healing patterns. *J Biomed Mater Res A* 2010; 93: 1358–1367.
19. Lei Y, Rai B, Ho KH, et al. In vitro degradation of novel bioactive polycaprolactone-20% tricalcium phosphate composite scaffolds for bone engineering. *Mater Sci Eng C Mater Biol Appl* 2007; 27: 293–298.
20. Dorozhkin SV. Bioceramics of calcium orthophosphates. *Biomaterials* 2010; 31: 1465–1485.
21. Bagambisa FB, Joos U and Schilli W. Mechanisms and structure of the bond between bone and hydroxyapatite ceramics. *J Biomed Mater Res* 1993; 27: 1047–1055.
22. Cao W and Hench LL. Bioactive materials. *Ceram Int* 1996; 22: 493–507.
23. Heughebart M, LeGeros RZ, Gineste M, et al. Physicochemical characterisation of deposits associated with HA ceramic implanted in nonosseous sites. *J Biomed Mater Res Appl Biomater* 1988; 22: 257–268.
24. Tracy BM and Doremus RH. Direct electron-microscopy studies of the bone-hydroxyapatite interface. *J Biomed Mater Res* 1984; 18: 719–726.
25. Arinzeh TL, Tran T, McAlary J, et al. A comparative study of biphasic calcium phosphate ceramics for human mesenchymal stem-cell-induced bone formation. *Biomaterials* 2005; 26: 3631–3638.
26. Ebrahimi M, Pripatnanont P, Monmaturapoj N, et al. Fabrication and characterization of novel nano hydroxyapatite/beta-tricalcium phosphate scaffolds in three different composition ratios. *J Biomed Mater Res A* 2012; 100: 2260–2268.
27. Ebrahimi M, Pripatnanont P, Suttapreyasri S, et al. In vitro biocompatibility analysis of novel nano-biphasic calcium phosphate scaffolds in different composition ratios. *J Biomed Mater Res Part B Appl Biomater* 2014; 102: 52–61.
28. Hahn BD, Park DS, Choi JJ, et al. Effect of the HA/β-TCP ratio on the biological performance of calcium phosphate ceramic coatings fabricated by a room-temperature powder spray in vacuum. *J Am Ceram Soc* 2009; 92: 793–799.
29. Lomelino Rde O, Castro S, Linhares II AB, et al. The association of human primary bone cells with biphasic calcium phosphate (betaTCP/HA 70:30) granules increases bone repair. *J Mater Sci Mater Med* 2012; 23: 781–788.
30. Tadashi S, Mitsuhiro S, Kouichi Y, et al. Influence of B-tricalcium phosphate dispersion on mechanical properties of hydroxyapatite ceramics. *J Ceram Soc Jpn* 2008; 116: 1002–1005.
31. Kokubo T, Kim HM and Kawashita M. Novel bioactive materials with different mechanical properties. *Biomaterials* 2003; 24: 2161–2175.
32. Bignon A, Chouteau J, Chevalier J, et al. Effect of micro- and macroporosity of bone substitutes on their mechanical properties and cellular response. *J Mater Sci Mater Med* 2003; 14: 1089–1097.
33. Gauthier O, Bouler JM, Aguado E, et al. Macroporous biphasic calcium phosphate ceramics: Influence of macropore diameter and macroporosity percentage on bone ingrowth. *Biomaterials* 1998; 19: 133–139.
34. LeGeros RZ. Properties of osteoconductive biomaterials: Calcium phosphates. *Clin Orthop elat Res* 2002; 395: 81–98.
35. Lee SH and Shin H. Matrices and scaffolds for delivery of bioactive molecules in bone and cartilage tissue engineering. *Adv Drug Deliv Rev* 2007; 59: 339–359.
36. Cheng L, Ye F, Yang R, et al. Osteoinduction of hydroxyapatite/beta-tricalcium phosphate bioceramics in mice with a fractured fibula. *Acta Biomater* 2010; 6: 1569–1574.
37. Mulliken JB, Kaban LB and Glowacki J. Induced osteogenesis-the biological principle and clinical applications. *J Surg Res* 1984; 37: 487–496.
38. Ripamonti U and Roden LC. Induction of bone formation by transforming growth factor-beta2 in the non-

human primate Papio ursinus and its modulation by skeletal muscle responding stem cells. *Cell Prolif* 2010; 43: 207–218.
39. Rueger JM, Siebert HR, Dohr-Fritz M, et al. Time sequence of osteoinduction and osteostimulation elicited by biologic bone replacement materials. *Life Support Syst* 1985; 3: 471–475.
40. Urist MR, Huo YK, Brownell AG, et al. Purification of bovine bone morphogenetic protein by hydroxyapatite chromatography. *Proc Natl Acad Sci USA* 1984; 81: 371–375.
41. Breitwieser GE. Extracellular calcium as an integrator of tissue function. *Int J Biochem Cell Biol* 2008; 40: 1467–1480.
42. Dvorak MM and Riccardi D. Ca2+ as an extracellular signal in bone. *Cell Calcium* 2004; 35: 249–255.
43. Orwar O, Lobovkina T, Gozen I, et al. Protrusive growth and periodic contractile motion in surface-adhered vesicles induced by Ca(2+)-gradients. *Soft Matter* 2010; 6: 268–272.
44. Nakade O, Takahashi K, Takuma T, et al. Effect of extracellular calcium on the gene expression of bone morphogenetic protein-2 and -4 of normal human bone cells. *J Bone Miner Metab* 2001; 19: 13–19.
45. Maeno S, Niki Y, Matsumoto H, et al. The effect of calcium ion concentration on osteoblast viability, proliferation and differentiation in monolayer and 3D culture. *Biomaterials* 2005; 26: 4847–4855.
46. Godwin SL and Soltoff SP. Extracellular calcium and platelet-derived growth factor promote receptor-mediated chemotaxis in osteoblasts through different signaling pathways. *J Biol Chem* 1997; 272: 11307–11312.
47. Duncan RL, Akanbi KA and Farach-Carson MC. Calcium signals and calcium channels in osteoblastic cells. *Semin Nephrol* 1998; 18: 178–190.
48. Dvorak MM, Chen TH, Orwoll B, et al. Constitutive activity of the osteoblast Ca2+ sensing receptor promotes loss of cancellous bone. *Endocrinology* 2007; 148: 3156–3163.
49. Dvorak MM, Siddiqua A, Ward DT, et al. Physiological changes in extracellular calcium concentration directly control osteoblast function in the absence of calciotropic hormones. *Proc Natl Acad Sci USA* 2004; 101: 5140–5145.
50. Beck G. Inorganic phosphate regulates multiple genes during osteoblast differentiation, including Nrf2. *Exp Cell Res* 2003; 288: 288–300.
51. Bingham PJ and Raisz LG. Bone growth in organ culture: effects of phosphate and other nutrients on bone and cartilage. *Calcif Tissue Res* 1974; 14: 31–48.
52. Liu YK, Lu QZ, Pei R, et al. The effect of extracellular calcium and inorganic phosphate on the growth and osteogenic differentiation of mesenchymal stem cells in vitro: Implication for bone tissue engineering. *Biomed Mater* 2009; 4: 025004.
53. Barrere F, van Blitterswijk CA and de Groot K. Bone regeneration: Molecular and cellular interactions with calcium phosphate ceramics. *Int J Nanomed* 2006; 1: 317–332.