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BIOLOGICAL BEHAVIOR OF BONE CELLS ON TWO TYPES OF HYDROXYAPATITE INCORPORATED POLYMER SCAFFOLD

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Oral Biology
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Thesis Title	BIOLOGICAL BEHAVIOR OF BONE CELLS ON TWO TYPES OF HYDROXYAPATITE INCORPORATED POLYMER SCAFFOLD
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บุณฑริกา ชื่นจิตกุลถาวร : พฤติกรรมทางชีววิทยาของเซลล์กระดูกบนวัสดุโครงร่างพอลิเมอร์สองชนิดที่มีส่วนประกอบของไฮดรอกซีแอปาไทต์. (BIOLOGICAL BEHAVIOR OF BONE CELLS ON TWO TYPES OF HYDROXYAPATITE INCORPORATED POLYMER SCAFFOLD) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ทพ.ดร. คำรงค์ คำรงค์ศรี, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ทพ.ดร. ประสิทธิ์ ภวสันต์, รศ.ดร. พิชญ์ ศุภผล 90 หน้า.

มาตรฐานที่ดีที่สุดในการรักษาการสูญเสียไปของกระดูกคือ การรักษาโดยใช้การปลูกถ่ายกระดูกของผู้ป่วยเอง แต่ปัญหาที่พบคือข้อจำกัดของแหล่งที่มาของกระดูกทดแทนที่ไม่เพียงพอ ดังนั้นทางคณะผู้วิจัยจึงใช้หลักการของวิศวกรรมเนื้อเยื่อกระดูกเพื่อพัฒนาวัสดุผสมที่มีคุณสมบัติในการสลายตัวได้ในทางชีวภาพ เพื่อการรักษารอยโรคของกระดูก ลำดับแรกคณะผู้วิจัยได้สร้างวัสดุโครงร่างจากพอลิแอลแลคติคแอซิด (พีแอลแอลเอ) ผสมด้วยไฮดรอกซีแอปาไทต์ (เอชเอพี) โดยวิธีการผลิตเส้นใยด้วยกระบวนการไฟฟ้า ทางคณะผู้วิจัยได้ทำการตรวจวัดลักษณะรูปร่างและคุณสมบัติทางกลของวัสดุโครงร่างนี้ด้วยกล้องจุลทรรศน์อิเล็คตรอนแบบส่องกราดและเครื่องมือทดสอบแบบเอนกประสงค์ตามลำดับ สำหรับการทดสอบทางชีววิทยาคณะผู้วิจัยได้ตรวจสอบความเป็นพิษต่อเซลล์และการแสดงออกของยีนที่เกี่ยวข้องทางชีววิทยากระดูก จากผลการศึกษาพบว่ามีการเพิ่มขึ้นของการแสดงออกของยีนออสตีโอแคลซินในระดับเอ็มอาร์เอ็นเอ และมีการสะสมของแร่ธาตุเมื่อทำการเลี้ยงเซลล์ก่อนการสร้างกระดูกของหนู (เอ็มซีสามทีสาม-อีหนึ่ง) กับวัสดุโครงร่างพีแอลแอลเอผสมเอชเอพี จากการพิจารณาคุณสมบัติทางกลและการตอบสนองทางชีววิทยาเป็นการยืนยันได้ว่าวัสดุพีแอลแอลเอผสมเอชเอพีที่มีลักษณะเป็นเส้นใย สามารถเป็นอีกหนึ่งทางเลือกของวัสดุที่จะนำมาใช้ในการสร้างใหม่ของกระดูก

วัสดุโครงร่างพีแอลแอลเอผสมเอชเอพีได้ผลการศึกษาเป็นที่น่าพอใจและวิธีการผลิตเส้นใยด้วยกระบวนการปั่นด้วยไฟฟ้าสามารถสร้างวัสดุโครงร่างที่มีลักษณะเป็นเส้นใยและมีรูพรุน แต่อย่างไรก็ตามวัสดุชนิดนี้ก็มีข้อจำกัดในเรื่องของรูปร่างและความหนา ดังนั้นคณะผู้วิจัยจึงได้พัฒนาวัสดุโครงร่างพอลิคาโปรแลคโตนผสมเอชเอพีที่ขึ้นรูปจากเทคนิคการหล่อแบบด้วยสารละลายและใช้สารที่ทำให้เกิดความพรุน จากวิธีนี้ทำให้สามารถควบคุมขนาดของรูพรุนได้ระหว่าง 400-500 ไมโครเมตร และสร้างรูปร่างและขนาดของวัสดุโครงร่างด้วยแบบหล่อที่เหมาะสม สำหรับในการวิเคราะห์ในห้องปฏิบัติการ เซลล์สร้างกระดูกจะถูกเลี้ยงร่วมกับวัสดุโครงร่างสามมิติชนิดนี้ โดยพบว่าพีซีแอลผสมเอชเอพีสามารถส่งเสริมการเพิ่มจำนวน เพิ่มการแสดงออกของคอลลาเจนชนิดที่หนึ่งและออสตีโอแคลซิน เอ็มอาร์เอ็นเอ และการสะสมแร่ธาตุได้ เมื่อเปรียบเทียบกับวัสดุโครงร่างพีซีแอลเพียงอย่างเดียวในการทดลองการสร้างใหม่ของกระดูกในสัตว์ทดลอง คณะผู้วิจัยได้ฝังวัสดุโครงร่างพีซีแอลผสมเอชเอพี ในรอยโรคที่ทำขึ้นในกระโหลกศีรษะของหนู และพบว่าเมื่อเวลาผ่านไปหกสัปดาห์ในรอยโรคที่มีพีซีแอลผสมเอชเอพีมีกระดูกสร้างขึ้นใหม่มากกว่ารอยโรคที่มีพีซีแอล จากผลการทดสอบในห้องปฏิบัติการและในสัตว์ทดลองแสดงให้เห็นว่าวัสดุโครงร่างพีซีแอลผสมเอชเอพีสามารถเป็นวัสดุสังเคราะห์ที่เหมาะสมสำหรับงานวิศวกรรมเนื้อเยื่อกระดูก อย่างไรก็ตามในการศึกษาเพิ่มเติมในอนาคตนั้นคณะผู้วิจัยจะทำการพัฒนาวัสดุชนิดนี้โดยการเติมปัจจัยการเติบโตและพัฒนาการของเซลล์และทำการทดสอบในสัตว์ทดลองชนิดอื่นต่อไป

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KEYWORDS : BONE TISSUE ENGINEERING / POLY(L-LACTIC ACID) / POLYCAPROLACTONE / HYDROXYAPATITE / 3D SCAFFOLD

BOONTHARIKA CHUENJITKUNTAWORN : BIOLOGICAL BEHAVIOR OF BONE CELLS ON TWO TYPES OF HYDROXYAPATITE INCORPORATED POLYMER SCAFFOLD. ADVISOR : ASST. PROF. DAMRONG DAMRONGSRI, D.D.S., Ph.D., CO-ADVISOR : ASSOC. PROF. PRASIT PAVASANT, D.D.S., Ph.D., ASSOC. PROF. PITT SUPAPHOL, Ph.D., 90 pp.

The gold standard for bone loss treatment is autograft but the problem is insufficient bony source. Therefore we used bone tissue engineering concept to develop biodegradable composite materials for repairing bony defect. First we constructed hydroxyapatite (HAp) incorporated poly (L-lactic acid) (PLLA) by electrospinning technique. We determined morphology and mechanical properties of these scaffold by scanning electron microscope and universal testing machine respectively. For biological testing, we evaluated cytotoxicity and bone biological marker gene expression. From the results we found the increasing of osteocalcin mRNA expression and mineralization in MC3T3-E1, a mouse pre-osteoblastic cell line, culturing on PLLA/HAp scaffolds. The results from mechanical properties and biological responses indicated that the fibrous PLLA/HAp could be the material of choice for use in bone regeneration.

The PLLA/HAp scaffolds had satisfiable results and electrospinning technique could construct the fibrous and porous scaffold. However, the drawback of this method is difficulty of producing for different shape and thickness. Therefore we developed Polycaprolactone/HAp (PCL/HAp) scaffold prepared by solvent casting and particulate leaching technique. From this procedure we could create porous size between 400-500 μm. and produeced the appropriated shape and size of the scaffold by proper model. For *in vitro* analysis, the primary bone cells were cultured with these three dimensional (3D) scaffolds. PCL/HAp promoted proliferation, increased collagen type I and osteocalcin mRNA expression and also mineralization when compared to PCL scaffolds. For *in vivo* bone regeneration, we implanted the PCL/HAp scaffolds in calvarial mice defect for 6 weeks. The PCL/HAp increased new bone formation when compared to that of PCL. The results from *in vitro* and *in vivo* testings showed that the PCL/HAp scaffold could be suitable synthetic material for bone tissue engineering. Further studies in the future, however, may include adding some growth factors and testing with other animal models.

Field of Study : Oral Biology Student's Signature Boontharika Chuenjitkuntaworn

Academic Year : 2009 Advisor's Signature .....

Co-Advisor's Signature .....

Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

3D	Three dimensional
ALP	Alkaline phosphatase
CAD	computer-aid design
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EDX	energy dispersive X-ray spectroscopy
HAp	Hydroxyapatite
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide
NaOH	Sodium hydroxide
PBS	Phosphate buffer saline
PCL	Polycaprolactone
PGA	Polyglycolide
PLA	Poly-D, L-lactic acid
PLLA	Poly (L-lactic acid)
RP	Rapid prototyping
RT-PCR	Reverse-transcription polymerase chain reaction
SCPL	Solvent casting and particulate leaching technique
SEM	Scanning electron microscope
SFF	solid free form fabrication
TCPS	Tissue culture plates

CHAPTER I

INTRODUCTION

Autograft from cortical or cancellous bone has been a gold standard for treatment of skeletal defects resulting from injury, trauma, disease, tumor and congenital abnormalities. Although it provides a source of biologically active tissue, its limit in bone supply, time-consuming surgery and complication due to donor site morbidity remain medical concerns. Allografts and xenografts are more readily available and provide tissue growth but, however, they are less biologically active and may carry the potential risk of disease transmission and immune rejection (Hutmacher 2000; Kujala et al., 2003; Hee et al., 2006).

Implant is one treatment of choices for replacing or restoring function in tooth and bone loss. However, some problems can be found with the treatment due to implant-related factor such as type and shape of material, surface topography, and surface chemistry. Mechanical loading, surgical technique, and patient variables such as bone quantity and quality may also cause some failure in dental implant as well (Puleo and Nanci, 1999). Therefore, increasing in bone quality by adopting some synthetic biomaterials could solve the problems and also provide a solution for the problems of autologous and allogous bone in skeletal tissue reconstruction.

Tissue engineering is an interesting area for promoting human health care administration, in which the basic understanding of the application of cellular biology and bioengineering for developing feasible substitutes to aid in the clinical treatment (Yang et al., 2005). For success in tissue reconstruction we focused on the concept of tissue engineering. There are three factors involved in this field including cells, engineering materials, and suitable biochemical factors to improve or replace biological function. For bone tissue engineering, a possible solution was the using of suitable three-dimensional scaffold that pre-seeded with bone cells or another source of cells for example mesenchymal stem cells. This stem cells have been shown to be multipotent cell lineages including fibroblastic, adipogenic, osteogenic, chondrogenic, and myogenic cells so called cell-based approach (Rose and Oreffo, 2002).

A variety of biomaterials, in particular polymers, have been investigated for their suitability in tissue engineering applications. Bone graft materials can be made from naturally-derived or synthetic materials. In the past decade, synthetic

biodegradable polymers have been continuously developed as bone graft substitutes. The most commonly synthetic polymer is Poly-L-lactic-acid (PLLA). It is widely used as material for surgical implants and clinically used as a bioresorbable suture material. PLLA is a promising scaffold material because of its biocompatibility and biodegradability properties (Ahmed and Tsuchiya, 2004). Polyglycolide (PGA) and polycaprolactone (PCL) from various fabrication techniques, along with their corresponding copolymers have also been tested as scaffolding materials for skin, cartilage and bone (Ciapetti et al., 2003; Kweon et al., 2003). Moreover, PCL has a slow degradation rate and can be engineered to degrade at a rate that will slowly transfer load to the healing bone. Thus biodegradable polymer may be developed for using in bone scaffolding applications. In this research we designated two types of polymer, PLLA and PCL, for apply as a scaffold in our experiments.

On characterization of scaffold material, surface topography and surface chemistry modifications were two major strategies to improve biocompatibility and cell growth of scaffold materials. Therefore, scientists have been refined the fabrication technologies of synthetic materials to get a proper three-dimensional (3D) structure. Porosity (both percentage and size) and interconnections within the scaffold, as well as the degradation rate of the polymer are parameters governing the adhesion of cells and the development of tissue. Several different techniques have been introduced to fabricate the porous conventional biodegradable polymer matrices but most of these techniques still have problems in controlling fiber diameter and alignment. As an alternative, the fiber spinning technique, or so called electrospinning, has been developed and succeeded in fabricating fibrous scaffolds with appropriated sizes and dimensions depending upon the field of application (Yang et al., 2005). For surface topography, electrospun scaffold has advantages over film or membrane sheet because it has interconnected pores and high porosity that resembles fibrous collagen in the natural extracellular matrix (Wutticharoenmongkol et al., 2007).

Recent studies focus on using the scaffolds prepared by various advanced techniques in order to create the scaffolds that precisely mimic a natural tissue in the human body. Solvent casting and particulate Leaching (SCPL) is such a technique that can create porous 3D scaffold structure. A porous form with interconnected channels for cell penetration is a crucial characteristic of biocompatibility scaffold material (Chaudhry et al., 2004). The scaffolds that prepared from this technique provide a

supporting frame and act as a template for osteogenesis. Moreover, the porous size can be controlled by the porogen size in the polymer.

Combination of two or more materials has been introduced and developed as a new strategy to improve some properties of 3D biodegradable polymer. For surface chemistry, various bioceramic fillers can be incorporated into porous polymer matrices in order to improve their mechanical and biological of the materials (Meretoja et al., 2006). In addition, many researchers have reported that incorporation of calcium carbonate ($CaCO_3$) or a type of calcium phosphate such as hydroxyapatite (HAp) helped to improve osteoblast proliferation and differentiation (Kim et al., 2004; Yoshikawa and Myoui, 2005). The combination with HAp enhanced the hydrophilicity and thus accelerated the biodegradation rate of nanofibrous film. Furthermore, improving nanofibrous scaffolds with HAp showed the great promise in cell culture condition and suggested these composite materials could apply in bone tissue engineering (Ito et al., 2005; Rouahi et al., 2006)

From the above reasons we created the new scaffold from PLLA with electrospinning and PCL incorporated with HAp with SCPL. We hypothesized that our composite scaffold could induce bone cells attachment, proliferation and mineralization. This study was undertaken to investigate biological abilities of the scaffold both in vitro and in vivo.

RESEARCH OBJECTIVES

1. To determine the effect of electrospun PLLA/HAp and SCPL PCL/HAp scaffold on proliferation of osteoblasts in vitro.
2. To determine the effect of electrospun PLLA/HAp and SCPL PCL/HAp scaffold on osteocalcin mRNA level in osteoblasts.
2. To determine the ability of osteoblasts on nodule formation and calcium precipitation after seeding in electrospun PLLA/HAp and SCPL PCL/HAp scaffold.
3. To study *in vivo* biological responses of PCL/HAp scaffold.

RESEARCH HYPOTHESIS

1. Three-dimensional electrospun PLLA/HAp and SCPL PCL/HAp scaffolds can improve proliferation of osteoblasts.
2. Osteoblasts can produce mRNA of osteocalcin after seeding in the electrospun PLLA/HAp and SCPL/HAp scaffold.
3. Osteoblasts can produce nodule formation and calcium precipitation after seeding in the scaffold.
4. PCL/HAp scaffold has acceptable biological responses *in vivo*.

EXPECTED BENEFIT

We anticipate that the results from this investigation would provide novel information regarding the development three-dimensional scaffolds. These new porous composite biomaterials may give a promising result as an alternative material for bone regeneration. We hope to use tissue engineering approach with this scaffold to replace damaged bone tissue in the future.

CHAPTER II
REVIEW OF RELATED LITERATURE

Bone is a true nanocomposite structure. It is a complex of highly specialized form of connective tissue relevant to perform the skeleton of the body. Bone provides not only mechanical support but also serves as a reservoir for minerals particularly calcium and phosphate (Murugan and Ramakrishna, 2005). Many people may have bone defects either from traumatic or non-traumatic destruction such as diseases and congenital abnormalities. The need for bone grafts depends on the nature and complication of the bone defects. If the defect is minor, bone has its own capability to self-regenerate within a few weeks and surgery is not required. However, in the case of severe defects and loss of large volume, bone can not heal by itself and the grafting is required to restore function. To define the requirements for bone grafting, it is necessary to assess the quality of the remaining bone. Preoperative examinations including multiplanar radiographs, computed tomography, or three-dimensional (3D) images are important to define the bone loss and the bone graft requirement (Goldberg, 2000). Autogenous bone grafts are the most effective graft material. Moreover, the large bone deficiencies usually exhibit failure of autograft transplantation and desire the use of allografts. Although autografting and allografting are clinically considered as good therapy, they have limitations. Therefore, at present there is still a great need for synthetic bone grafts.

Bone grafts

Autologous bone graft is a method in which tissue or organ is transplanted from one site to another site of the same individual. It may be obtained from the greater trochanter, the anterior and posterior iliac crest. However, it has disadvantages in some aspects: inadequate supply, donor-site morbidity and the procedure prolongs the time of surgery (Goldberg and Stevenson, 1987).

Allogenous bone graft or human graft is transferred from a donor to a recipient of the same species but nonidentical genetic composition. Therefore it can carries the potential risk of disease transmission and immune rejection (Reynolds and Bowers, 1996; Mokbel et al., 2008).

Xenogenous bone graft is derived from a donor of a different species such as bovine bone. It is made from donor bone which the organic components are totally removed to avoid immunological reactions. On the other hand, the remaining inorganic substances still provide a natural architectural matrix. However, the problem of using this graft is quantity and cost (Richardson et al., 1999).

Synthetic composite materials currently used for reconstruction bone loss area including metals, alloys, ceramics and polymers. In most of the cases, metals and ceramics are used in hard tissue applications, while polymers in soft tissue applications due to their mechanical properties. Composites are widely used in both the applications. Evolution of biomaterials in bone grafting is illustrated in Figure 2.1. These materials using in fabrication of tissue-engineered bone have some significant advantages such as providing structural integrity within the body and eventually be able to break down at intended period.

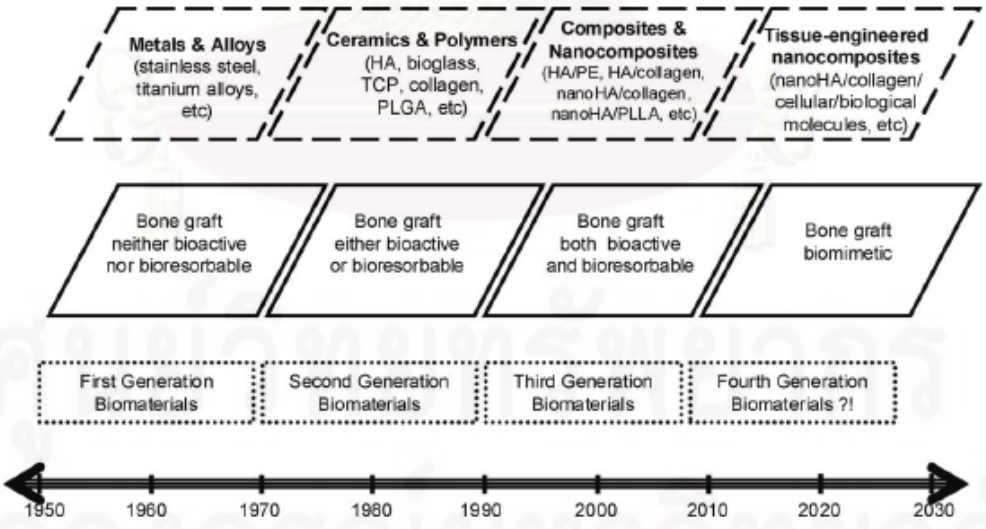


Figure 2.1 Evolution of biomaterials in bone grafting (from Murugan and Ramakrishna, 2005).

Function of Bone Graft

Before considering the desired features of potential bone graft materials, it is useful to understand two concepts in bone regeneration for tissue-engineering; they are osteoconduction and osteoinduction

Osteoconduction is a function of a bone graft that provides a three-dimensional structure that acts as a framework for capillaries and osteoprogenitor cells ingrowth and support bone forming processes (Goldberg and Stevenson, 1987). An osteoconductive material guides repair in a location where normal healing occurs if left untreated. Many of available bone graft substitutes are able to contribute new bone formation by this biological process. For example, the tubular absorbable polymers used in long bone defects are believed to maintain an osteogenic-rich medullary environment within the defect and therefore allowing direct bone growth onto the polymer skeleton (Goldberg, 2000).

Osteoinduction is defined as the ability in recruitment of pluripotential cells, from a non-osseous environment to differentiate into chondrocytes and osteoblasts in bone formation. An osteoinductive material allows repair in a location that would normally not heal if left untreated (Burg et al., 2000). It is mediated by graft-derived factors such as bone morphogenetic proteins and other growth factors.

Bone grafts could serve two functions, osteogenesis and mechanical support. Osteogenesis is the bone formation process occurs either from cells of the graft or from cells of the host. For mechanical function, bone graft could provide structural support for host bone either as a primary function or as a result of the remodeling of the original graft (Goldberg, 2000). Nowadays, the new technologies using tissue engineering may provide bone graft materials serve both functions and efficiency for bone regeneration.

Tissue engineering

Tissue engineering is a concept that composed of three components including cells, engineering materials or scaffold to support the growth and differentiation of the cells and the suitable biochemical factors to improve or replace biological functions (Figure 2.2).

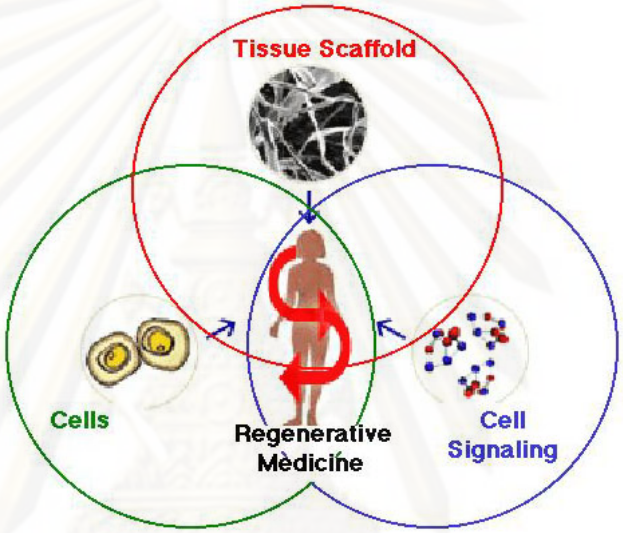


Figure 2.2 Component of tissue engineering.
(from http://tissueengineering.com/images/platform_technologies_diagram.gif)

The scaffold

The biomaterial scaffolds typically function as a three-dimensional (3D) structural support, which facilitate tissue integration into the skeletal defect and promote cell attachment, proliferation, and differentiation. The 3D scaffold will provide a supporting frame and act as a template for osteogenesis. Porous structure with interconnected channels is crucial factor because the penetration of nutrient could support survival of bone cells. The required characteristics of the material for the scaffold also include biocompatibility and biodegradability (Chaudhry et al., 2004).

Scaffolds should serve at least one of the following purposes:

1. Allow cell attachment and migration.
2. Deliver and retain cells and biochemical factors.
3. Enable diffusion cell nutrients and expressed products to vital
4. Exert certain mechanical and biological influences to modify the behavior of the cell phase.

Degradable is one of the essential properties for the scaffolds because the scaffolds need to be absorbed by the surrounding tissues without the necessity of a surgical removal. The degradation rate should correlated with the rate of tissue formation: this means that while cells are fabricating their own natural matrix structure around themselves, the scaffold is able to provide structural integrity within the body and eventually break down leaving newly formed tissue which will be ready to take over the mechanical load.

A variety of natural and synthetic scaffold materials have already been investigated for bone tissue engineering, for example, naturally-derived materials included fibrin, hyaluronic acid, collagen gels and sponges. The commonly used synthetic materials are degradable polymers such as polylactides and polyglycolides. Ceramics is another group of synthetic materials including hydroxyapatite, tricalcium phosphate, coral and bioactive glass (Alexander et al., 2008).

Synthetic biodegradable polymers have been used extensively for scaffold fabrication in tissue engineering applications. These polymers can differ in their molecular weight, polydispersity, crystallinity, and thermal transitions, allowing different in degradation rate. Their relative hydrophobicity, percentage of crystallinity and variation in surface charge will affect cellular spreading or affinity for the surface, which can also cause change in phenotypic expression. Good biocompatibility and possibility to process into desired configurations bring to their popularity. A desirable contour of engineered tissue was achieved by the synchronization of polymer degradation and natural tissue replacement (Sung et al., 2004; El-Amin et al., 2006).

Biodegradable polymer

Poly-D, L-lactic acid (PLA)

Lactide is the cyclic dimer of lactic acid, which exists as two optical isomers, D and L. L-lactide is the naturally occurring isomer, and DL-lactide is the synthetic blend of D-lactide and L-lactide. The polymerization of lactide showed in figure 2.3. The homopolymer of L-lactide (PLLA) is a semicrystalline polymer. It exhibits high tensile strength and low elongation and consequently have a high modulus that makes them applicable for load-bearing applications such as in orthopedic fixation and sutures. Much more rapid degradation time making it more attractive as a drug delivery system. PLLA hydrolyzes to lactic acid which is a normal product of muscular contraction in animals. The lactic acid is then further metabolized through the tricarboxylic acid cycle and then excreted as carbon dioxide and water (Middleton and Tipton, 2000).

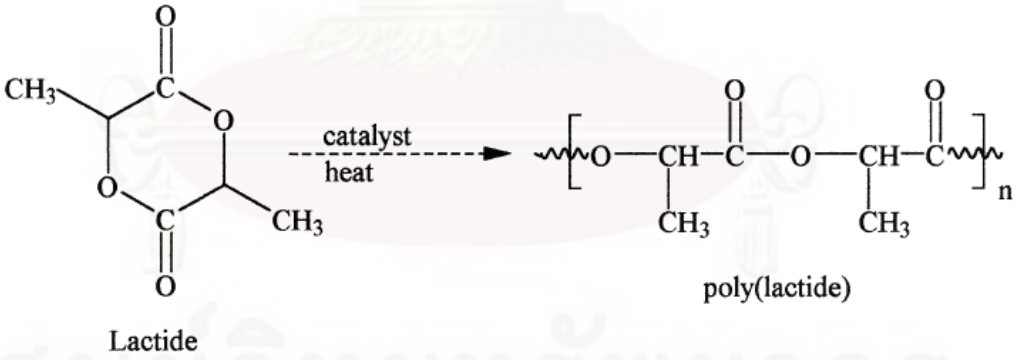


Figure 2.3 Synthesis of poly-D, L-lactic acid (PLA) (from Middleton and Tipton, 2000).

From Alexander et al., 2008, they studied human jaw periosteal cells (JPC) growing within open-cell polylactic acid (OPLA) scaffolds and showed the higher proliferation rates and formation of nodules after initiation of osteogenesis among other 3D collagen composites.

Polycaprolactone (PCL)

PCL is a semicrystalline linear resorbable aliphatic polyester. It has biodegradation property because of the susceptibility of its aliphatic ester lingkage to hydrolysis. PCL can be prepared by ring opening polymerization of ε-caprolactone using a catalyst such as stannous octanoate (Figure 2.4). PCL is degraded by hydrolysis of its ester linkages in physiological conditions (such as in the human body) and has therefore received a great deal of attention for use as an implantable biomaterial. In the body, the products generated are either metabolized via the tricarboxylic acid (TCA) cycle or eliminated by direct renal secretion.

In vitro and in vivo biocompatibility and efficacy studies have been performed, and the results made US FDA approval of PCL to use as medical and drug delivery devices. At present, PCL has been used as a soft and hard-tissue compatible material including resorbable suture, drug delivery system, and recently bone graft substitutes (Pena et al., 2006). The PCL was attractive due to its low cost, sustained biodegradability, and availability at low molecular weight. PCL is more stable in ambient condition, it is significantly less expensive and is readily available in large quantities (Marletta et al., 2007). Currently, PCL is regarded as a candidate polymer for tissue engineering, as it shows sufficient mechanical properties to serve as a scaffold in applications where a more resilient material is required, e.g. bone substitution, where the physical properties of the scaffold have to be maintained for at least 6 months (Agrawal and Ray, 2001).

Figure 2.4 Ring opening polymerization of ε-caprolactone to polycaprolactone (from http://www.Synthetic Biodegradable Polymers as Medical Devices (MPB archive, Mar 98).html).

In particular, PCL is especially interesting materials for the preparation of long term implantable devices, owing to its degradation which is even slower than that of polylactide. Examination of cell-seeded constructs by scanning electron microscopy (SEM) provided the visual confirmation of good PCL/cell interaction. In fact, when culture SaOS-2, an osteoblastic cell line, on the surfaces of the PCL materials for 3-4 weeks the surfaces of PCL were found to be gradually covered by the cells. More than one single cell layer was recognized with cytoplasm of different cells often merging to each other. The cells showed a polygonal shape with no regular orientation. Previous study has showed that human osteoblasts were attached and proliferated on PCL networks. Their proliferation index (PI), however, were decreased compared with cells cultured in cultured plates with significant difference, suggesting that PCL network is not yet sufficient for bone marrow conductance and needs additional matrix for cell attachment and supporting cell proliferation (Kweon et al., 2003).

In PCL implantation experiment, a common finding was the presence of thin, capillary-like processes extending from one cell to another and to material structures. Quantification was made using SEM images, but some differences between PCL samples were recorded: small and large mineralized nodules were more easily observed onto stimulated body fluid-treated PCL samples compared to untreated PCL surfaces. Therefore PCL sample alone was not enough for bone cells differentiation (Ciapetti et al., 2003).

PCL scaffold was constructed in many methods, for example, electrospinning. Among these PCL composites tested (PCL, PCL/$CaCO_3$ and PCL/HAp), scaffold made from electrospun PCL/HAp composite fibers showed the best result when tested in vitro with L929 and SaOS-2 because of the known ability of HAp to promote bone-cell activities (Wutticharoenmongkol et al., 2006).

Hydroxyapatite (HAp)

HAp and other bioactive calcium phosphate materials have received considerable attention as materials for implants and bone augmentation procedures. They are used as a bone replacement because they chemically bond to bone when implanted, resulting in the formation of a strong bone-implant interface (Verma et al., 2006). HAp has extended interest as a drug delivery carrier due to its osteoconductivity and biocompatibility. In addition it has been proved that HAp can enhance osteoblast differentiation as well as osteoblast growth (osteoconductive property).

HAp: $Ca_{10}(PO_4)_6(OH)_2$ has been extensively investigated over the past few decades as a biomedical material. It mimics the natural apatite composition of bone and teeth that possesses the same chemical composition (Ca/P=1.67). HAp has been designated as a bioactive material, meaning that it has osteoconductive property and forms a strong bond to natural bone in vivo. A common method of improving the mechanical properties of polymers is to incorporate fillers into polymers. There are two advantages of choosing HAp as the filler material for preparing polymeric-biomaterial scaffolds: (1) HAp acts as a reinforcing material to improve the mechanical properties of the scaffold; (2) HAp can improve the osteoconductivity of the scaffold.

Practically, the porous forms of HAp have been used as bone scaffolds to improve bone ingrowth and osseointegration (Yoshikawa and Myoui, 2005). However, HAp cannot be easily shaped into the bone defect sites and it is too brittle. The brittleness limited its extended applications for hard tissue implants. On the other hand, synthetic biodegradable materials are of significance in improving the mechanical properties of such materials, if used together.

However, the effect of HAp on tissue response has not yet been fully understood. It is known that the cellular responses depend upon the physical and chemical characteristics of the substrate such as crystallinity, particle size and surface structure (Deligianni et al., 2001). HAp is arranged within a collagen matrix forming a supportive framework (Di Silvio et al., 2002). The presence of HAp particle together with the deposition of a Ca-P rich layer onto the scaffold surface provides ions for

mineralization. The synthesis of a composite polymer plus bonelike materials, including HAp or tricalcium phosphate (TCP) particles, is a recognized technique for reinforcement of the polymeric structure.

Calcium phosphate materials, in particular HAp, have been shown to undergo different degrees of degradation. The HAp releases calcium and phosphate ions that may influence the nearby cell population thus enhancing the bone apposition and bonding to bony tissue (Rouahi et al., 2006). Moreover, the additional advantage of Ca-P salts is the ability to buffer the acidic by-products from degradation. The dissolution characteristics imply that HAp may serve as a source of inorganic phosphate to enhance bone cell mineralization.

Techniques for scaffold preparation

Three biodegradable synthetic scaffold materials have been approved by The US Food and Drug Administration (FDA) including polylactic acid (PLA), polyglycolic acid (PGA), and polycaprolactone (PCL). PLA is commonly used synthetic material because it degrades within the human body to form lactic acid, a naturally-occurring chemical which is easily removed from the body. Similar materials are PGA and PCL; their degradation mechanism is similar to that of PLA, but they exhibit a faster and a slower rate of degradation compared to PLA, respectively. Nowadays, the material scientists are refining the synthesis of materials and fabrication technologies to get a proper 3D structure. Porosity (percent and size) and interconnections within the scaffold, as well as the degradation rate are parameters governing the adhesion of cells and the development of tissue. Therefore, several different techniques have been introduced to fabricate the porous conventional biodegradable polymer matrices. These techniques were list as followed.

1. Electrospinning

This technique is driven by electrostatic field to control the formation and deposition of polymer nanofibers that requires only small amounts of polymer (figure 2.5). The procedure, which is technically feasible for fabrication of filaments ranging in the nanometer to micrometer scale with a certain degree of alignment, is remarkably

efficient, rapid and inexpensive (Srouji et al., 2006). This process can produces highly porous non-woven fabrics consisting of ultrafine fibers. A wide variety of polymers have been constructed by electrospinning technique. Several applications such as drug delivery and implant materials have been proposed in recent years based on the small fiber diameters and high porosities characteristic. The fiber diameters that are attainable in the electrospinning process depend on the polymer and the process conditions. Due to the small fiber diameters and the overall porous structure, electrospun fabrics have a high specific surface area that is beneficial for tissue engineering and biomedical applications. It is possible to manipulate them into 3D structures during their deposition (Deitzel et al., 2001). The features of nanofiber mats are morphologically similar to extracellular matrix (ECM) of the natural tissue which are characterized by a wide range of pore diameter distribution, high porosity, effective mechanical and specific biochemical properties (Venugopal et al., 2008). Recently, the electrospinning method has attracted a great deal of attention to produce non-woven membranes of nanofibers. From previous study the PCL/HAp composite in the form of electrospun fiber mats could support osteogenic differentiation of MC3T3-E1. The potential for use of the PCL and PCL/HAp fiber mats as bone scaffolds was thoroughly evaluated in terms of the attachment, proliferation, differentiation, and mineralization of MC3T3-E1 (Wutticharoenmongkol et al., 2007). Electrospun biomimetic PLLA incorporated with collagen and hydroxyapatite (PLLA/collagen/HAp) nanofibers hold great potential for adhesion, proliferation and mineralization of osteoblasts (Prabhakaran et al., 2009). Therefore the electrospinning method can construct biocomposite scaffolds that may be suitable for bone tissue regeneration.

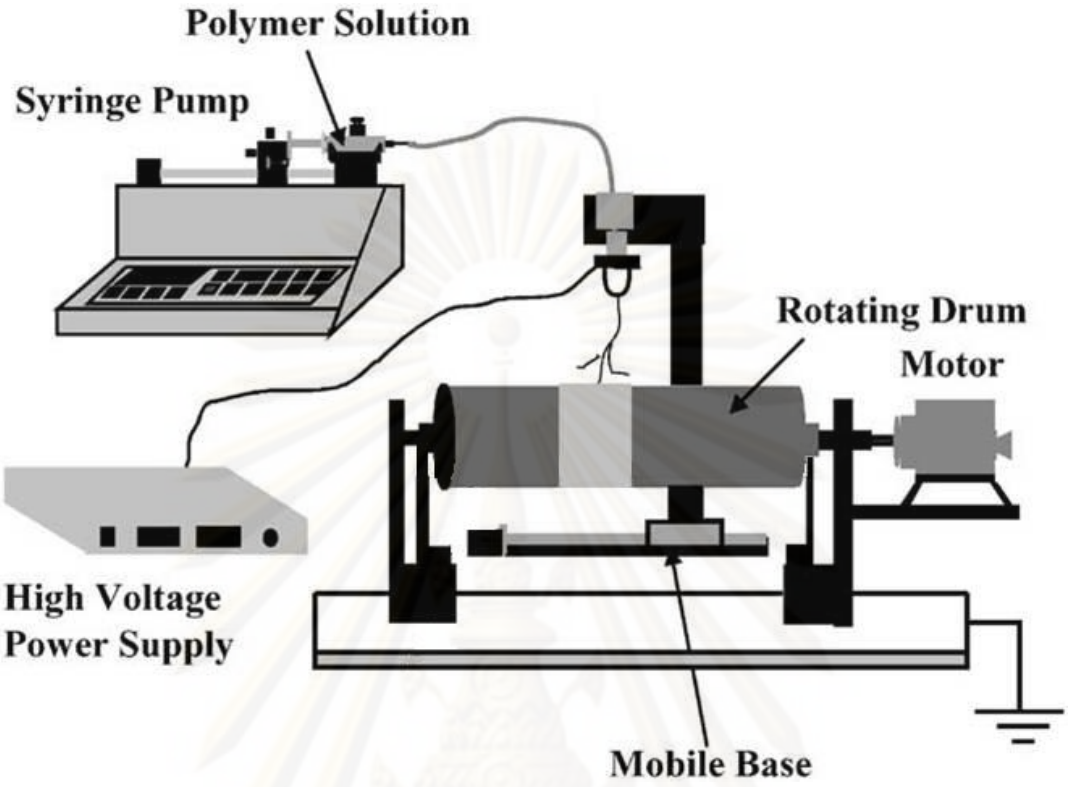


Figure 2.5 Schematic representation of the electrostatic fiber spinner. An electric field is generated by applying a high voltage between the metal capillary and the collector. The polymer fluid is delivered to the capillary at a constant flow rate. The jet thins away from the nozzle and subsequently bends and whips. This instability region is indicated by the shaded region (from Zong et al., 2002).

2. Emulsification/Freeze-drying

This technique constructs the scaffold without the use of solid porogen. First, a synthetic polymer is dissolved into a suitable solvent (e.g. polylactic acid in dichloromethane) then water is added to the polymeric solution and the two liquids are mixed in order to obtain an emulsion. Before the two phases separate, the emulsion is cast into a mold and quickly frozen by means of immersion into liquid nitrogen. The frozen emulsion is subsequently freeze-dried to remove the dispersed water and the solvent, thus leaving a solidified, porous polymeric structure. This technique allows a faster preparation and using appropriate solvents. The small pore size and irregular

porosity are among its disadvantages. However, freeze-drying technique is still a commonly employed technique for the fabrication of specific scaffolds, in particular collagen sponges.

3. Rapid prototyping (RP)

This technique is also known as solid free form fabrication (SFF). RP techniques involve building 3D objects using layered manufacturing methods. The process, in general, comprises the design of a scaffold model using computer-aid design (CAD) software, which is then presented as a series of cross-sections. Corresponding to each cross-section, the RP machine lays down a layer of material starting from the bottom and moving up a layer at a time to create the scaffold. Each new layer adheres to the one below it, thereby providing integrity to the finished product. The main advantage of RP technique is its ability to finely control the microstructure and macrostructure of scaffolds and thus produce complex topographies from a computer model. It can control porosity and pore size. Therefore computer assisted design and this manufacturing technique has been introduced to fabricate tissue engineering materials (Karande et al., 2004; Williams et al., 2005).

4. Solvent Casting and Particulate Leaching (SCPL)

This approach allows the preparation of porous structures with regular porosity and can control porous size. First, the polymer is dissolved into a suitable organic solvent (e.g. polylactic acid could be dissolved into dichloromethane, polycaprolactone in chloroform) then the solution is cast into a mold filled with porogen particles. Such porogen can be an inorganic salt like sodium chloride, crystals of saccharose, gelatin spheres or paraffin spheres. After the polymer solution has been cast the solvent is allowed to fully evaporate, then the composite structure in the mold is immersed in a bath of a liquid suitable for dissolving the porogen. The liquid is water in case of the porogen are sodium chloride, saccharose and gelatin or an

aliphatic solvent like hexane for paraffin. Once the porogen has been fully dissolved a porous structure is obtained. The size of the porogen particles will affect the size of the scaffold pores, while the polymer to porogen ratio is directly correlated to the amount of porosity of the final structure. Various bioceramic fillers can be incorporated into porous polymer matrices to improve their mechanical properties or to bring bioactivity to the material (Meretoja et al., 2006). Other than the small thickness range that can be obtained, another drawback of SCPL lies in its use of organic solvents which must be fully removed to avoid any possible damage to the cells seeded on the scaffold.

Porous size

It is well recognized that the pore size of scaffolds plays an important role for cell binding, migration and tissue ingrowth and regeneration. Generally, the cells need the pore diameter to be large enough to allow them to migrate into porous scaffold. The porous size should be large enough to allow effective nutrient supply and metabolic waste removal which are essential for effective cell growth, but should be small enough to establish a sufficiently high surface area for efficient binding of the scaffold and cell–cell interactions for better cell growth (Philips et al., 2006; Oh et al., 2007).

Pore size is responsible for cellular behavior and also cell-type specific. Since bony archetecture has very different structure depending on its function and location, bring to the reason that the same pore shape may not be ideal for all potential uses (Burg et al., 2000). The different types of cells showed different growth behaviors in the scaffolds; the fibroblasts showed best cell growth in the scaffold section having 186–200 μm., while the chondrocytes and osteoblasts showed better cell growth in the scaffold sections having larger pore sizes. The exact mechanism of different growth behavior of different types of cells in the scaffolds with different pore size is not clear yet (Coombes et al., 2004).

There are many studies try to determine the appropriate pore size for different cell types used in bone tissue engineering. Phillips et al. in 2006 reported that the pore shape has profound effect on the attachment and long-term survival of cells on a surface and optimal pore topography that can be readily modulated by careful

selection porogen. The scaffold should also have interconnected macropores from 100 to 500 µm. in diameter to allow vascularization and good penetration of tissue. While another study indicated that the optimal range of pore size for bone ingrowth into porous metals is often reported as 50-400 µm. Structures characterized by pore sizes larger than 150 µm are generally preferred because they provide a suitable environment for bone reorganization and vascularisation. However, excellent bone ingrowth has been reported in structures with larger pore sizes than 400 µm. indicating that the optimal pore size for bone ingrowth is structure dependant (St-Pierre et al., 2005).

It was observed that the cells and tissue prefer different pore size ranges in the scaffolds for effective cell growth and tissue regeneration: 380–405 µm pore size for chondrocyte and osteoblast growth (Oh et al., 2007). Logeart-Avramoglou et al., 2005 proposed that pore sizes in the range of 200-900 µm have performed most satisfactorily in bone tissue regeneration because, in addition to osteoprogenitor cells, they also enable endothelial cells to migrate into the matrix and develop the vascular beds necessary to nourish the newly form tissue as well. Solvent casting and particulate leaching technique is capable of modulate the pore topography and size to suit a particular cell type, e.g., osteoblasts. From SCPL method we can construct 3-D scaffold that provides the necessary support for cells to proliferate and maintain their differentiated function, and its architecture defines the ultimate shape of the new bone and cartilage. From all of the above we decided to construct 400-500 µm. porous scaffold in this experiment because it is within the optimum range reported in several previous studies.

Composite materials for bone tissue engineering

From biomimetic standpoint, various types of biological tissue such as bone are composite tissues with organic and inorganic phases, various cell types, extracellular matrix and bone mineral (Lu et al., 2005). Since single existing material possesses limitation in some necessary properties required for an ideal bone graft, there is growing interest in development of composite materials.

The composite is expected to have improved mechanical properties, compared to the elaborate polymer, and better structural integrity and flexibility more than ceramics only. HAp, though brittle by itself, has been used for bone regeneration and biomedical implant applications due to its biodegradability, bioactivity and osteoconductive properties. Many studies demonstrated that the characteristics of composite scaffold were superior more than separate materials (summarized in table 2.1).

Table 2.1 Previous studies of composite materials.

Composite materials	Fabrication techniques	References
PLLA/HAp	Electrospinning	Sui et al., 2007
PLA/silica based bioactive glass	Electrospinning and Thermal pressing	Kim et al., 2008
PLA/collagen/HAp	Electrospinning	Prabhakaran et al., 2009
PLA/HAp	Solvent casting and particulate leaching	Kothapalli et al., 2005
PCL/HAp	Electrospinning	Wutticharoenmongkol et al., 2007
PCL/HAp	Emulsification and freeze-drying	Causa et al., 2006
PCL/HAp	Rapid prototyping	Heo et al., 2009
PLA/HAp and PCL/HAp	Solvent casting	Rizzi et al., 2001
PCL/HAp	Solvent casting	Chim et al., 2006

The incorporation of calcium phosphate glass particles to the PLA foams improved both, mechanical properties and biological response of the scaffolds. When the glass particles are introduced into the 3-D structure of the polymer foam, the pore walls get more rigid and as a consequence, they do not bend and collapse as easily as in the case of the polymer foams. Moreover, the PLA/glass foam showed higher cell viability and proliferation values than the PLA foam. Thus, these results suggest that the glass particles in the scaffold had a positive influence in the cell (Navarro et al., 2004). Therefore, a possible strategy to enhance mechanical properties is to reinforce PCL scaffold with rigid HAp particles, which would also improve osteoconductivity of the polymer (Rizzi et al., 2001; Chim et al., 2006). A preliminary study on HAp incorporated in PCL scaffolds with a continuous cell line has demonstrated that the presence of HAp on PCL substrates enhanced the osteoblast function and growth (Causa et al., 2006).

Biocomposite nanofibers containing HAp fabricated by electrospinning might be another suitable scaffold with improved biological properties for bone tissue engineering. The possibility of fabricating a nano-biomaterial based scaffold suitable for cellular proliferation with a strength matching natural bone. The electrospun PLLA/collagen/HAp nanofibers is one of promising structural scaffolds because of its biocompatibility, osteoconductivity and sufficient mechanical strength for bone tissue engineering (Prabhakaran et al., 2009). A nanocomposite constituted of the silica-based bioactive glass nanofiber and PLA, which was developed for use as the bone regeneration matrix, showed the good bioactivity, including the precipitation of bone-like apatite mineral on its surface. Osteoblastic cells showed favorable cell attachment and proliferation behavior on the nanocomposite (Kim et al., 2008).

The appropriate scaffold not only have biodegradable properties but also perform a high surface volume ratio, in other words, they should have a highly porous structure. New PCL scaffolds with interconnected spherical pores and 70% porosity can regenerated cartilage *in vitro* and *in vivo* as well (Izquierdo et al., 2008). Moreover, HAp incorporated PLA 3D scaffolds with 90% porosity and interconnectivity using solvent-casting/salt-leaching method demonstrated that 50% by weight of HAp incorporation in the scaffold showed 150% increased in yield strength and doubled the compressive modulus when compared to scaffold made with pure PLA (Kothapalli et al., 2005). The 3D scaffolds made of nano HAp/PCL

composites were fabricated with 72-73% porosity and 400-500 μm showed improving both compressive modulus value and attachment and proliferation of MG-63 cells (Heo et al., 2009). Therefore, the development of appropriate composite scaffold that fabricated from proper composition and technique could bring about the better mechanical and biological properties for bone tissue engineering than polymer or bioceramic material alone.

CHAPTER III
ELECTROSPUN POLY(L-LACTIC ACID)/ HYDROXYAPATITE COMPOSITE FIBROUS SCAFFOLDS FOR BONE TISSUE ENGINEERING

Summary

Background: Poly(L-lactic acid) (PLLA) is one of the most studied synthetic biodegradable polymeric materials as bone graft substitutes. Due to the osteoconductive property of hydroxyapatite (HAp), we prepared fibrous matrices of PLLA without and with the presence of HAp particles in the amount of 0.25 or 0.50% (w/v, based on the volume of the base 15% w/v PLLA solution in 70:30 v/v dichloromethane/tetrahydrofuran). These fibrous matrices were assessed for their potential for use as substrates for bone cell culture.

Results: The presence of HAp in the composite fiber mats was confirmed by energy dispersive X-ray spectroscopy mapping. The average diameters of both the neat PLLA and the PLLA/HAp fibers, as determined by scanning electron microscopy, ranged between 2.3 and 3.5 μm, with the average spacing between adjacent fibers ranging between 5.7 and 8.5 μm. The porosity of these fibrous membranes was high (i.e., ~97-98%). The direct cytotoxicity evaluation with L929 mouse fibroblasts indicated that the neat PLLA fiber mat released no substance in the level that was toxic to the cells. The presence of HAp particles at 0.50% w/v in the PLLA fibrous scaffold not only promoted the attachment and the proliferation of MC3T3-E1 mouse pre-osteoblastic cells, but also increased the expression of osteocalcin mRNA and the extent of mineralization after the cells had been cultured on the scaffold for 14 and 21 d, respectively.

Conclusion: The obtained results suggested that the PLLA/HAp fiber mats could be materials of choice for bone tissue engineering.

Introduction

Bone grafts have been used to repair skeletal defects caused by injuries, trauma, diseases and congenital abnormalities. The ideal treatment for such defects is by the use of autografts, commonly harvested from iliac crest. It is, however, limited by availability and morbidity of the donor sites. The use of allografts or xenografts is hampered by potential risks of transmitted diseases and immune responses of the host (Hutmacher 2000 ; Hutmacher et al., 2000; Kujala et al., 2003). Based on these reason, the use of synthetic materials as bone graft substitutes has become a viable choice. Among such materials, poly(L-lactic acid) (PLLA), a polymer derived from L-lactic acid or L-2-hydroxypropionic acid, is classified as an biocompatible and biodegradable polyester. Due to its unique properties, PLLA is one of the most studied materials in its class (Kenawy et al., 2002; Badami et al., 2006; Chen et al., 2006; Kim et al., 2006; Sui et al., 2007; Sangsanoh et al., 2007; Jeong et al., 2008) and has been used extensively in medicine, e.g., as surgical suture (i.e., as a copolymer thread with glycolic acid for normal healing wounds or as a pure polymer thread for wounds that require a healing time of up to 28 weeks (Heino et al., 1996), suture anchor, etc.

Tissue engineering is an interdisciplinary field that applies both engineering and life science principles towards the development of biological substitutes that can restore, maintain and/or improve tissue functions (Langer and Vacanti, 1993). A wide variety of fabrication techniques have been used to generate three dimensional (3D) polymeric scaffolds for potential applications in tissue regeneration. Electrospinning, a processing technique capable of producing ultra-fine fibers with diameters in sub-micrometer down to nanometer range through the action of a high electric field, is one such technique. Under a high electric field, a stream of a polymer liquid (solution or melt) is ejected towards a collector. It undergoes a significant stretching with simultaneous evaporation of solvent within or cooling of the ejected jet, resulting in the deposition of solid fibers in the form of a non-woven fabric on the collector (Reneker and Yarin, 2008). Even though the morphology of the electrospun polymeric fiber mats is, to some extent, similar to that of the collagen bundles in the natural extracellular matrix (ECM), the typical structure of the electrospun fiber mats is

usually 2D in nature (Teo et al., 2006) (viz. due to the relatively smaller inter-fibrous pore sizes as compared to those of the cells). In spite of this limitation, the unique characteristics of the electrospun fiber mats, such as high surface area, high porosity and high inter-pore connectivity, make them interesting candidates as scaffolds for tissue engineering (Badami et al., 2006; Kim et al., 2006; Teo et al., 2006; Wutticharoenmongkol et al, 2006, 2007; Sangsanoh et al., 2007; Sui et al. 2007).

Synthetic hydroxyapatite (HAp) is a ceramic material, most often used in combination with a scaffold for bone regeneration because of its biocompatibility properties (Zambonin and Grano, 1995; Murugan and Ramakrishna, 2006). The presence of HAp particles in a scaffold mimics the apatitic phase of the bone, while the deposition of a Ca-P rich layer onto the scaffold surface provides ions for mineralization. HAp exhibits excellent resorbable and osteoconductive properties, thus it enhances bone apposition and facilitates the bonding of the scaffold to the surrounding bony tissue (Rouahi et al., 2006). Previously, successful fabrication of electrospun polycaprolactone (PCL) fiber mats containing calcium carbonate ($CaCO_3$) or HAp particles was reported. These materials were proven to be non-toxic and acted as good supports for the attachment and the proliferation of human osteogenic sarcoma cells (SaOS-2), especially for the one that had been filled with 1 wt.% HAp (i.e., PCL/HAp-FS) (Wutticharoenmongkol et al., 2006). The full osteoblastic expression of the mouse calvaria-derived pre-osteoblastic cells (MC3T3-E1) that had been cultured on the surface of PCL/HAp-FS was also proven to be a result of the presence of HAp (Wutticharoenmongkol et al., 2007).

With regards to electrospun PLLA fiber mats that incorporate HAp particles, a limited number of published articles are available in the open literature (Kim et al., 2006; Jeong et al., 2008). These reports provided certain details about physico-chemical (i.e., dispersion of HAp particles within PLLA fibrous matrix, specific surface area, average pore diameter, *in vitro* degradation in phosphate buffer and thermal stability) and mechanical properties of the PLLA/HAp fibrous matrices. In terms of biological evaluation, these matrices have been used as substrates for the cultures of MG-63 osteosarcoma cells or MC3T3-E1. The viability of MG-63 that had been cultured on these matrices for up to 7 d was evaluated spectrophotometrically (Kim et al., 2006; Sui et al., 2007) while the ability of MC3T3-E1 to attach on the

matrices was evaluated by observing the organization of cytoskeleton. A long-term culture of MC3T3-E1 was also evaluated for up to 21 d (Jeong et al., 2008). Despite the observed increase in the number of the proliferated cells (based on double-strand DNA quantification), no direct study with regard to the expression of protein markers specific to bone cell differentiation nor biomineralization of MC3T3-E1 that had been cultured on the matrices was evaluated. Notwithstanding, only the expression of alkaline phosphatase, an early bone protein marker, was evaluated for MG-63 that had been cultured on the matrices for 7 day.

In the present contribution, electrospun PLLA and PLLA/HAp fiber mats were fabricated. The materials were characterized for their morphology, mechanical integrity, porosity and cytotoxicity. The potential for use of the electrospun PLLA and PLLA/HAp fiber mats as substrates for bone cell culture was thoroughly evaluated in terms of the attachment, proliferation, expression of mRNA specific for the production of certain bone proteins, and biomineralization of MC3T3-E1 that had been either seeded or cultured on their surfaces. Comparisons were made with the cells that had either been seeded or cultured on a tissue-culture polystyrene plate (TCPS) and, in some cases, solvent-cast PLLA films. Even though the concept for the fabrication of the electrospun PLLA/HAp fiber mats as substrates for bone cell cultures is not new, the present contribution provides convincing evidence on the effect of HAp on the proliferation and differentiation of the cultured MC3T3-E1 at the biological level.

Experimental details

Materials

Materials used for the fabrication of the fibrous scaffolds were poly(L-lactic acid) (PLLA; M_n = 200,000 g·mol^{-1}, intrinsic viscosity = 0.332 dL·g^{-1}), dichloromethane (Carlo Erba, Italy), tetrahydrofuran (Carlo Erba, Italy) and hydroxyapatite particles (HAp; synthesized based on the method proposed by Shih et al., 2004. The mean size of the as-synthesized HAp particles was analyzed by a JEOL JSM 5410LV scanning electron microscope (SEM) to be 234 ± 68 nm. (Wutticharoenmongkol et al., 2006).

Preparation and characterization of scaffolds

Two types of PLLA scaffolds were fabricated: electrospun PLLA fiber mats and solvent-cast PLLA films (internal control). For the electrospun PLLA fiber mats, either neat PLLA or PLLA/HAp fiber mats were fabricated. The content of HAp in the composite fibrous materials was either 0.25 or 0.50% (w/v of the PLLA solution), which was equivalent to either 1.67 or 3.33% (w/w of PLLA). Hereafter, the as-prepared PLLA/HAp fiber mats were denoted as PLLA/HAp0.25% and PLLA/HAp0.50%, respectively. The word ‘scaffold’ is used here to represent a substrate of any topographical character that is used for cell culturing purposes.

The electrospun PLLA and PLLA/HAp fiber mats were prepared from 15% w/v PLLA solution in 70:30 v/v dichloromethane/tetrahydrofuran (Sangsanoh et al., 2007) and the base PLLA solutions containing HAp particles at either 0.25 or 0.50% w/v, respectively. Briefly, the base PLLA solution or each of the PLLA/HAp suspensions was contained in a glass syringe, the open end of which was connected to a blunt 20-gauge stainless steel hypodermic needle, used as the nozzle. An aluminum (Al) sheet wrapped around a rotating cylinder (OD ≈ 15 cm; rotational speed = 50 rpm) was used as the collector. A Gamma High-Voltage Research ES30P-5W high-voltage power supply was used to generate a high dc potential, with the positive, emitting electrode being connected to the nozzle and the grounding one to the collector. The electrospinning was conducted with an electrical potential of 15 kV that was applied over a collection distance of 10 cm for a collection time of 5 h. The thicknesses of the obtained fiber mats were measured by means of a Mitutayo IP65

micrometer to be 125 ± 5 μm. On the other hand, the PLLA films were prepared by casting 5% w/v PLLA solution in chloroform in glass Petri dishes. The films were left in a fume hood for 1 d to allow solvent evaporation. The thicknesses of the films were also measured by the micrometer to be 120 ± 5 μm. Both the fiber mats and the films were incubated *in vacuo* at 40 °C to remove as much solvent as possible.

Morphology of the fibrous and the film scaffolds was examined by SEM. At least 200 readings for the diameters of the individual fiber segments within the fibrous scaffolds taken from at least 5 SEM images were statistically analyzed using a SemAphore 4.0 software. Inter-fiber spacings (D_s) between the individual fiber segments within the fibrous scaffolds could be calculated based on a knowledge of the number of fibers (N) within a viewing area (A), according to an empirical model of the form: $D_s = 0.5/\sqrt{N/A}$ (Sanders et al., 2005). The porosities of the fibrous scaffolds (ε) were estimated based on the difference between the density of PLLA (ρ_{PLLA}) (that is, about 1.191 g·cm^{-3}) and the bulk densities of the fiber mats (ρ_{SC}) according to an equation of the form: $\varepsilon(\%) = (1-\rho_{PLLA}/\rho_{SC})\times 100$ (Wutticharoenmongkol et al., 2006). The bulk densities of the fiber mats (ρ_{SC}) were investigated by a Sartorious YDK 01 density measurement kit based on the knowledge of the weights of the fiber mat specimens that were measured either in air (w_a) or in water (w_w) and the density of water (ρ_w; that is, 0.99648 g·cm^{-3} @ 27.2 °C), based on the equation of the form: $\rho_{SC} = w_a\rho_w / 0.99983(w_a - w_w) + 0.0012$.

The presence of HAp in the electrospun PLLA/HAp0.50% fiber mats was characterized qualitatively by energy dispersive X-ray spectroscopy (EDX; Link ISIS series 300) mapping. The actual content of HAp in the electrospun PLLA/HAp fiber mats was evaluated by gravimetric method. Briefly, samples of around 1 g were separately heated from room temperature to 800 °C at a rate of 10 °C·min^{-1} and held at 800 °C for 5 h in an oxygen atmosphere, using a Caresf ESF 12125 Carbolizer oven. Mechanical integrity of the fibrous scaffolds in terms of the yield and the tensile strengths was investigated using a Lloyd LRX universal testing machine (gauge length = 50 mm and crosshead speed = 10 mm/min) on the specimens that had been cut into a rectangular shape (10 mm × 70 mm). The widths and lengths of the specimens were measured by a graduated ruler and the thicknesses by the micrometer. Lastly, the static contact angles of water drops on the surfaces of the fibrous scaffolds were analyzed by

a Krüss DSA 10-MK2 drop shape analyzer. Specifically, water drops of a constant volume of ~18 μL had been placed on the surfaces of the fibrous scaffolds in various positions and allowed to sit on the surfaces for 2 min prior to the measurements.

Cytotoxicity evaluation

The potential for use of the PLLA fiber mats and the PLLA films (internal control) as substrates for tissue/cell culture was evaluated with a direct cytotoxicity method, using mouse fibroblasts (L929) as reference cells. First, L929 were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (FBS; Biochrom AG, Germany), 1% L-glutamine (Invitrogen, USA) and 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate and amphotericin B (Invitrogen, USA)]. Each of the fibrous and the film scaffolds was cut into circular discs (~15 mm in diameter), and the disc specimens were placed in empty wells of a 24-well TCPS (Corning, USA), which were later sterilized in 70% ethanol for 60 min. The specimens were then washed with autoclaved, deionized water and subsequently immersed in MEM overnight. To ensure a complete contact between each specimen and the bottom of each well, a sterilized stainless steel 316 ring (~12 mm in diameter) was placed on top of each specimen. L929 were then seeded on the specimens and empty wells of TCPS (positive control) at ~50,000 cells/cm^2 and maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for either 24 or 48 h. Finally, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was carried out to quantify the viability of the cells. The experiment was carried out in triplicate.

The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals relates to the number of viable cells in a linear manner. First, each cell-cultured specimen was incubated with 250 μL/well of MTT solution (i.e., 0.5 $mg \cdot mL^{-1}$ in DMEM without phenol red) at 37 °C for 45 min. After incubation, the MTT solution was removed and a buffer solution containing dimethylsulfoxide (DMSO; Carlo Erba) (900 μL/well) and glycine buffer (pH = 10) (125 μL/well) was added to dissolve the formazan crystals. After 10 min of agitation, each sample solution was then transferred into a cuvette and placed in a Thermospectronic Genesis10 UV-vis spectrophotometer, from

which the absorbance at 570 nm representing the viability of the cells was measured. It should be emphasized that cells in various biological stages (e.g., unattached, attached, proliferated or differentiated stage) exhibit different mitochondria's activities, which could lead to different MTT absorbance values.

Cell attachment and cell proliferation

The potential for use of the neat PLLA and the PLLA/HAp fiber mats as substrates for bone cell culture was evaluated with mouse calvaria-derived pre-osteoblastic cells (MC3T3-E1). First, MC3T3-E1 were cultured in minimum essential medium with Earle's balanced salts (MEM; Hyclone, USA), supplemented with 10% FBS, 1% L-glutamine and 1% antibiotic and antimycotic formulation, as previously mentioned. Similarly, the sterilized fibrous scaffold specimens were placed in wells of a 24-well TCPS and secured with a sterilized metal ring, as previously mention. MC3T3-E1 were then seeded on the specimens and empty wells of TCPS (positive control) at ~30,000 cells/cm^2 and maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for either 8 h (for the attachment study) or 24 h (for the proliferation study). Again, the viabilities of both the seeded and the cultured cells was quantified by the MTT assay and the increase in the MTT absorbance values observed at 24 h of cell culturing from those observed at 8 h of cell seeding signified the proliferation of the cells. These experiments were carried out in triplicate.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

MC3T3-E1 were cultured on the neat PLLA and the PLLA/HAp fibrous scaffolds as well as on the PLLA film (internal control) and TCPS (positive control). On day 2, 8 and 14 after cell culturing, total RNA was extracted using Tri Reagent (Molecular Research Center, USA). Chloroform (1 mL) was added to the homogenized specimens, followed by precipitation with 500 μL of isopropanol (Sigma, USA). RNA pellets were washed with 70% ethanol and were dissolved in 15 μL of nuclease-free water (Promega, USA). RNA yields were evaluated by the UV-vis spectrophotometer based on the absorbance at 260 nm. First strand DNA was reverse-transcribed from 1 μg of total RNA using RT kit (ImProm-II™ Reverse Transcription System; Promega, USA). For the amplification in PCR, the PCR mixture consisting of 1 μL of cDNA, sense primer, antisense primer and reagent of PCR kit (Tag DNA

Polymerase; Qiagen, USA) was used. Specific primers for type-I collagen (COL1A1 gene), connexin-43 (Tanaka et al., 2003), osteocalcin (Jarrahy et al., 2005) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH; internal control) (Tsukamoto et al., 1992) were used. Semi-quantitative PCR was performed at 24 cycles for GAPDH, 28 cycles for type-I collagen and 30 cycles for connexin-43 and osteocalcin. The PCR products were analyzed by separation on 1.8% agarose (Usb, USA) gel using electrophoresis (Power Pac Junior, Bio-Rad, USA) and visualized with ethidium bromide (EtBr; Bio-Rad, USA) staining. The stained bands were photographed under UV light and the intensity was quantified using Scion Image software. The experiment was carried out in triplicate.

It should be noted that type-I collagen, connexin-43 and osteocalcin are among the biological markers of osteoblastic differentiation. Type-I collagen is the major structural ECM protein of the bone matrix. Connexin-43 is the major gap junction protein in osteoblasts that is responsible for the cell-cell communication (Tanaka et al., 2003; Jarrahy et al., 2005). Osteocalcin is a noncollagenous protein synthesized by mature osteoblasts and plays an important role in mineralization (Murugan and Ramakrishna, 2006; Wuttichareon mongkol et al., 2007).

Mineralization analysis

Calcium deposition was quantified using alizarin red S (i.e., an anthraquinone derivative) staining. MC3T3-E1 had been cultured on the neat PLLA and the PLLA/HAp fibrous scaffolds for 21 d. On day 3 after cell culturing, the medium was changed to the one that contained ascorbic acid (50 $\mu g \cdot mL^{-1}$; Sigma, USA) and β-glycerophosphate (5 mM; Sigma, USA). On day 21 after cell culturing, the cells were fixed with cold methanol for 10 min and washed with deionized water prior to immersion for 3 min in 370 μL of 1% Alizarin Red S (Sigma, USA) solution in 1:100 (v/v) ammonium hydroxide/water mixture. Each of the stained specimens was washed several times with deionized water and air-dried at room temperature. The stained specimen was photographed and the stained area, signifying the amount of calcium deposition, was quantified by Scion Image software. The intensity for each specimen was subtracted by the intensity of the corresponding blank specimen (i.e., either the neat PLLA or the PLLA/HAp fibrous scaffold without the cell culture). The cultured specimens were also observed by SEM. The experiment was carried out in triplicate.

Morphological observation of cultured cells

After the removal of the culture medium, each cell-cultured fibrous scaffold specimen was rinsed with PBS twice and the cells were then fixed with 3% glutaraldehyde solution [diluted from 50% glutaraldehyde solution (Electron Microscopy Science, USA) with PBS] at 500 µL/well. After 30 min, it was rinsed again with PBS. After cell fixation, the specimen was dehydrated in graded ethanol solutions (i.e., 30, 50, 70 and 90%, respectively) and in pure ethanol for 2 min each. It was then dried in 100% hexamethyldisilazane (HMDS; Sigma, USA) for 5 min and finally air-dried. Finally, the specimen was mounted on a SEM stub, coated with gold and observed by SEM.

Statistical analysis

All values were expressed as mean ± standard deviation. Statistical analysis was carried out by the one-way analysis of variance (one-way ANOVA) and Sheffe's post hoc test in SPSS (SPSS, USA). Statistical difference between two sets of data was considered when $p < 0.05$.

Results and discussion

Morphological, physical, mechanical and wettability characteristics of scaffolds

Electrospun fibrous scaffolds could be ideal structural matrices that exhibit physical appearance resembling the fibrillar structure of collagen bundles in the ECM that play a crucial role in tissue regeneration (Teo et al., 2006). The use of the neat PLLA and the PLLA/HAp fiber mats for tissue engineering is an attempt to mimic the ECM and, due to the presence of osteoconductive HAp, the PLLA/HAp fiber mats could be ideal materials for bone tissue regeneration (Zambonin and Grano, 1995; Murugan and Ramakrishna, 2006; Rouahi et al., 2006; Wutticharoenmongkol et al., 2006, 2007).

Figure 3.1 shows selected SEM images of the neat PLLA and the PLLA/HAp0.50% fiber mats. Evidently, the obtained fibers were rather smooth with occasional presence of elongated beads. Though not shown, the morphology of the PLLA/HAp0.25% fiber mats was similar to those shown in Figure 3.1 b. The morphology of the PLLA films, on the other hand, was rather smooth, with an evidence of rough protrusions on some parts of the film surface (result not shown). The formation of these rough protrusions was due possibly to the uneven shrinkage of the films during the evaporation of the solvent. The diameters of the neat PLLA fibers were 3.5 ± 1.1 μm, while those of the PLLA/HAp0.25% and PLLA/HAp0.50% counterparts were lower at 2.3 ± 0.7 and 2.5 ± 0.8 μm, respectively. The smaller diameters of the PLLA/HAp composite fibers, in comparison with those of the neat PLLA ones, could be due to the increase in the restriction to flow due to the presence of the HAp particles. The inter-fiber spacing of the neat PLLA fiber mats was 8.5 μm on average, while those of the composite counterparts were respectively lower at 6.1 and 5.7 μm on average. These inter-fiber spacing values corresponded well with the fact that the PLLA/HAp fiber mats were denser than the PLLA counterparts. On the other hand, the porosity values of these fiber mats were not much different from one another. Such values for the neat PLLA, the PLLA/HAp0.25% and the PLLA/HAp0.50% fiber mats (as estimated from the difference between the density values of PLLA and the fiber mats) were 97.6 ± 0.2, 98.1 ± 0.3 and 97.4 ± 0.6%, respectively. The inclusion of HAp in the PLLA/HAp0.50% fiber mats was qualitatively confirmed by EDX mapping for the presence of calcium (see Figure 3.2). The actual contents of HAp in the PLLA/HAp0.25% and the PLLA/HAp0.50% fiber

mats, as determined by gravimetric method after the samples had been thermally treated at 800 °C to completely burn off the PLLA matrix, were 1.11 ± 0.08 and 2.32 ± 0.10% (w/w of PLLA), accounting to about 62 and 67% of the HAp contents initially loaded in the neat PLLA solution.

The mechanical integrity of these fibrous materials was also assessed. The yield and the tensile strengths of the neat PLLA fiber mats were 0.56 ± 0.07 and 0.50 ± 0.08 MPa, respectively, while the inclusion of the HAp resulted in a slight increase in both of the property values, with such values for the PLLA/HAp0.25% and the PLLA/HAp0.50% fiber mats being 0.56 ± 0.09 and 0.52 ± 0.06 MPa and 0.88 ± 0.07 and 0.65 ± 0.20 MPa, respectively. Obviously, the presence of the HAp stiffened the resulting composite fiber mats. Sui et al., 2007 reported that the tensile strength of the PLLA/HAp fiber mats (average diameter of the individual fibers = 313 nm) was greater than that of the neat PLLA counterparts (average diameter of the individual fibers = 368 nm) (i.e., 2.86 ± 0.24 MPa versus 1.40 ± 0.11 MPa). Similarly, Jeong et al., 2008 reported that the tensile strengths of the PLLA/HAp fiber mats with the HAp contents of 5 and 20% (w/w of PLLA) were 0.16 ± 0.01 and 0.26 ± 0.01 MPa (average diameters of the individual fibers = 255 and 135 nm, respectively), which were greater than that of the neat PLLA counterparts of 0.06 ± 0.00 MPa (average diameter of the individual fibers = 365 nm). The increase in the stiffness of the PLLA/HAp composite fiber mats should be due to the specific interaction between –COOH groups of PLLA and the Ca^{2+} ions of HAp (Sui et al., 2007).

All of the fibrous scaffolds were also characterized for their surface activity in terms of the wettability by static water contact angle measurements. Here, water drops (~18 μL) were placed on different areas of the fiber mat surfaces. The surface of the neat PLLA fiber mats appeared to be most hydrophobic, with the static water contact angles of 115 ± 1°. A slightly lower value of ~107° was reported in the literature for the neat PLLA fiber mats with the average diameter of the individual fibers being 1 μm. (Zong et al., 2005). The presence of HAp caused the surfaces of the composite fiber mats to be more hydrophilic, with the static water contact angles being 106 ± 4° and 101 ± 4° for the PLLA/HAp0.25% and the PLLA/HAp0.50% fiber mats, respectively. Notwithstanding, the fact that the diameters of the PLLA/HAp composite

fibers were smaller than those of the neat PLLA counterparts could also contribute to the observed lower static water contact angle values of the composite fiber mats.

Cytotoxicity evaluation

Figure 3.3 shows viability of L929 that had been cultured on both the neat PLLA fiber mat and the corresponding film specimens as well as empty wells of TCPS for 24 or 48 h. Evidently, the viability of the cells, as determined by the absorbance according to the MTT assay, on any type of the substrates was found to increase with an increase in the culturing time, a result that indicated the ability of the substrates to support the proliferation of the mouse fibroblasts. At any given time point, the viability of the cells that had been cultured on the fibrous substrate was greater than those of the cells that had been cultured on both the film and TCPS, while that of the cells that had been cultured on the film was lower than that of the cells that had been cultured on TCPS. Upon normalizing with the viability of the cells that had been cultured on TCPS at any given time point, the viability ratios of the cells that had been cultured on the fiber mat and the film substrates were ~109 and ~83% at 24 h after cell culturing, while they were ~120 and ~80% at 48 h after cell culturing. Since the viability ratios of the cells that had been cultured on the fiber mat and the film substrates at any given time point were greater than 80%, it can be concluded that both the fiber mat and the film specimens were non-toxic to the cells and could be further assessed for their potential for use as bone scaffolds.

Cell attachment and cell proliferation

The potential for use of the as-prepared fibrous scaffolds in supporting both the attachment and the proliferation of bone cells was assessed with MC3T3-E1. The cells were either seeded or cultured on the surfaces of the fibrous scaffolds and TCPS (positive control) for 8 or 24 h, respectively (Figure 3.4). Evidently, the viability of the attached cells, as determined by the absorbance according to the MTT assay, on the PLLA/HAp0.50% fibrous scaffold was similar to that on TCPS, while the attachment of the cells on the rest of the fibrous scaffolds was inferior to that on TCPS. Among the various substrates, the PLLA/HAp0.50% fibrous scaffold appeared to be the best in supporting the attachment of the pre-osteoblastic cells, followed by the PLLA/HAp0.25% and the PLLA fibrous scaffolds, respectively. For the proliferation

of the cells, both of the HAp-containing fibrous scaffolds were able to support the proliferation of the cells in similar levels to that on TCPS, while only the neat PLLA fibrous scaffold was inferior to both the PLLA/HAp fibrous scaffolds and TCPS. Despite the different ability of the cells to attach on these substrates, the proliferation rate of the cells (i.e., the ratio of the viability of the proliferated cells at 24 h after cell culturing to that of the attached cells at 8 h after cell seeding) on the neat PLLA and the PLLA/HAp0.50% fibrous scaffolds was equivalent to that on TCPS (i.e., 111-114%), while that of the cells on the PLLA/HAp0.25% fibrous scaffold showed a greater value (i.e., 142%).

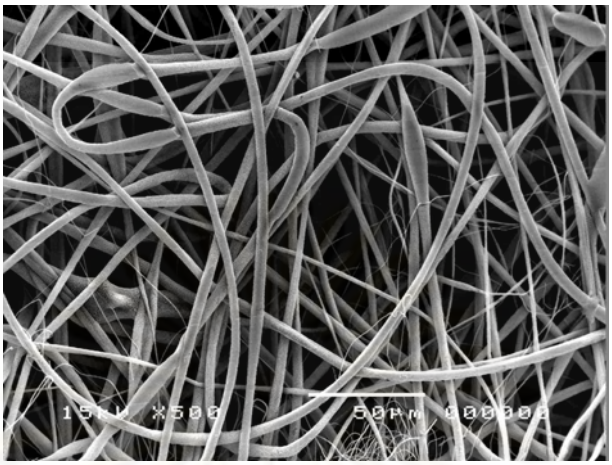
RT-PCR analysis

The expression of type-I collage, connexin-43 and osteocalcin mRNAs in MC3T3-E1 that had been cultured on the surfaces of the neat PLLA, the PLLA/HAp0.25% and the PLLA/HAp0.50% fibrous scaffolds as well as those of the PLLA film and TCPS for 14 d is shown in Figure 3.5. The expressed amounts of mRNAs in the cultured cells were semi-quantified by the band intensities, shown in Figure 3.5a. Apparently, the band intensities for the expression of the GAPDH mRNA for the cells that had been grown on different substrates were similar. Normalization of the band intensities associated with type-I collage, connexin-43 and osteocalcin mRNAs with those of GAPDH mRNA revealed more evidence on the relative amounts of the different mRNAs expressed in the cultured cells (see Figure 3.5b). Apparently, the expressed amounts of the investigated mRNAs in the cells that had been cultured on the fibrous scaffolds were significantly greater than those in the cells that had been cultured on TCPS and, particularly, the film counterpart. Interestingly, the expressed amount of osteocalcin mRNA in the cells that had been cultured on the PLLA/HAp0.50% fibrous scaffold was significantly greater than that in the cells that had been cultured on the PLLA/HAp0.25% and the PLLA fibrous scaffolds. While the increase in the expressed amounts of all of the investigated mRNAs in the cells that had been cultured on the fibrous scaffolds for 2 and 8 d was not significant, the expressed amount of osteocalcin mRNA in the cells that had been cultured on the fibrous scaffolds for 14 d was significantly increased from that observed on day 8 (results not shown). The significant increase in the level of the osteocalcin gene

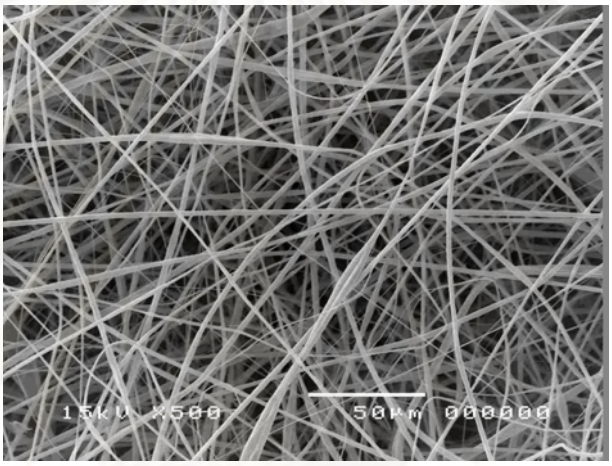
expression between days 8 and 14 suggested that the cells had entered the matrix maturation stage and were ready for subsequent mineralization (Wutticharoenmongkol et al., 2007).

Bone nodule formation and SEM observation

Alizarin Red S staining was used to characterize the bone nodule formation of MC3T3-E1 that had been cultured on the surfaces of the neat PLLA, the PLLA/HAp0.25% and the PLLA/HAp0.50% fibrous scaffolds for 21 d (see Figure 3.6). Figure 3.6a shows photographic images of the stained specimens. In the presence of calcium, the stained product (i.e., an Alizarin Red S-calcium chelating product) appeared red. Quantitative analysis of the results shown in Figure 6a was carried out by quantifying the number of the red pixels observed in each image and the results of such analysis are shown in Figure 3.6b. Clearly, the number of the red pixels for the PLLA/HAp0.50% fibrous scaffold was greater than those for the PLLA/HAp0.25% fibrous scaffold and, particularly, the PLLA fibrous scaffold. The results demonstrated clearly that the cells that had been cultured on the surface of the PLLA/HAp0.50% fibrous scaffold exhibited the most positive staining for calcium deposition, followed by those on the surfaces of the PLLA/HAp0.25% and the PLLA fibrous scaffolds, respectively. Mineralization of MC3T3-E1 that had been cultured on the fibrous scaffolds for 21 d was further characterized by SEM (see Figure 3.7). Evidence of the cultured cells and their ECM was observed on the surface of these fibrous scaffolds, with the underlying fibrous structure of the scaffolds being visible. In agreement with the results from the Alizarin Red S staining, the amount of the solid residue on the surface of the PLLA/HAp0.50% fibrous scaffold was significantly greater than that on the surface of the neat PLLA counterpart.



(a)



(b)

Figure 3.1. Representative SEM images of fiber mats electrospun from
(a) 15% w/v PLLA solution in 70:30 v/v dichloromethane/tetrahydrofuran and
(b) the PLLA solution containing 0.50% w/v of HAp particles under the electric field of 15 kV/10 cm.

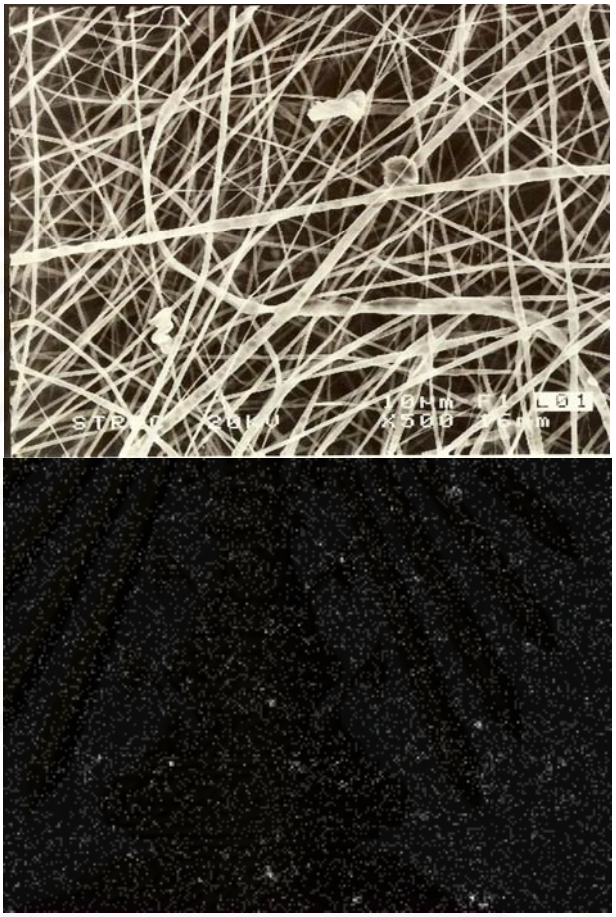


Figure 3.2. Representative SEM image and its corresponding EDX image for calcium of the fiber mat electrospun from the PLLA solution containing 0.50% w/v of HAp particles under the electric field of 15 kV/10 cm.

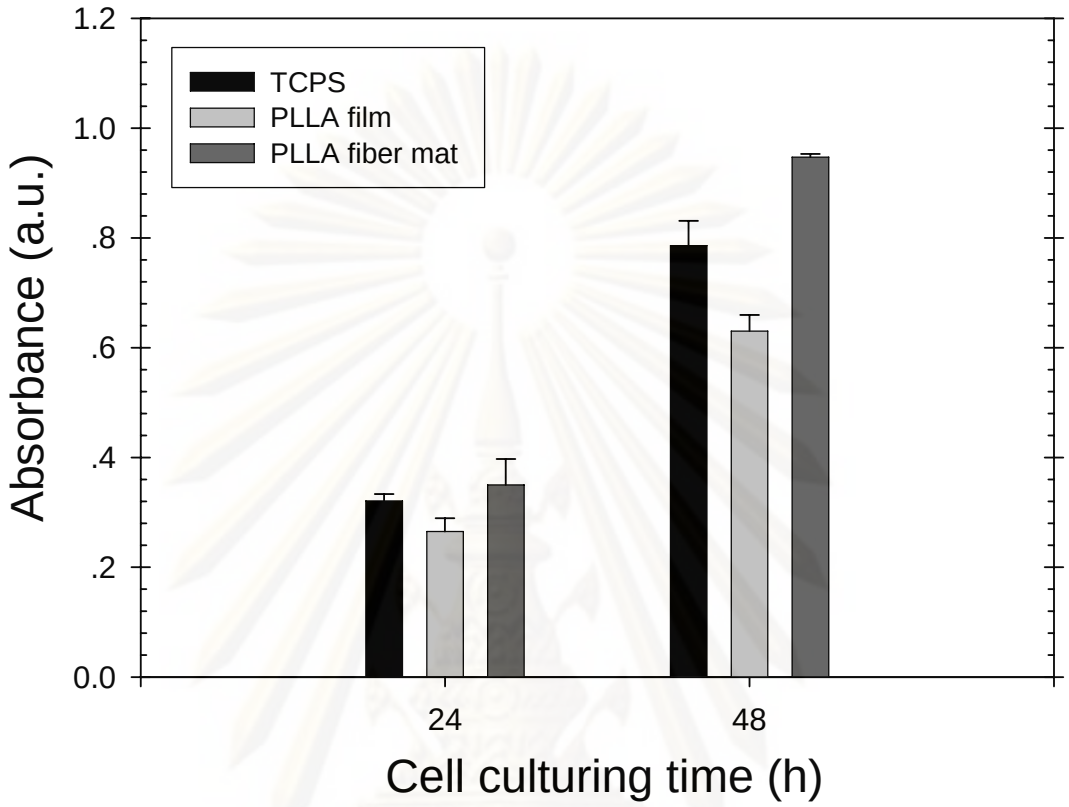


Figure 3.3. Direct cytotoxicity evaluation of the electrospun PLLA fiber mat and the solvent-cast PLLA film (internal control). L929, as the reference cells, were cultured on the surfaces of the fiber mat and the film specimens in comparison with those cultured on tissue-culture polystyrene plate (TCPS; positive control). The viability of the cultured cells was determined at 24 and 48 h after cell culturing. No significance at $p < 0.05$ with respect to TCPS.

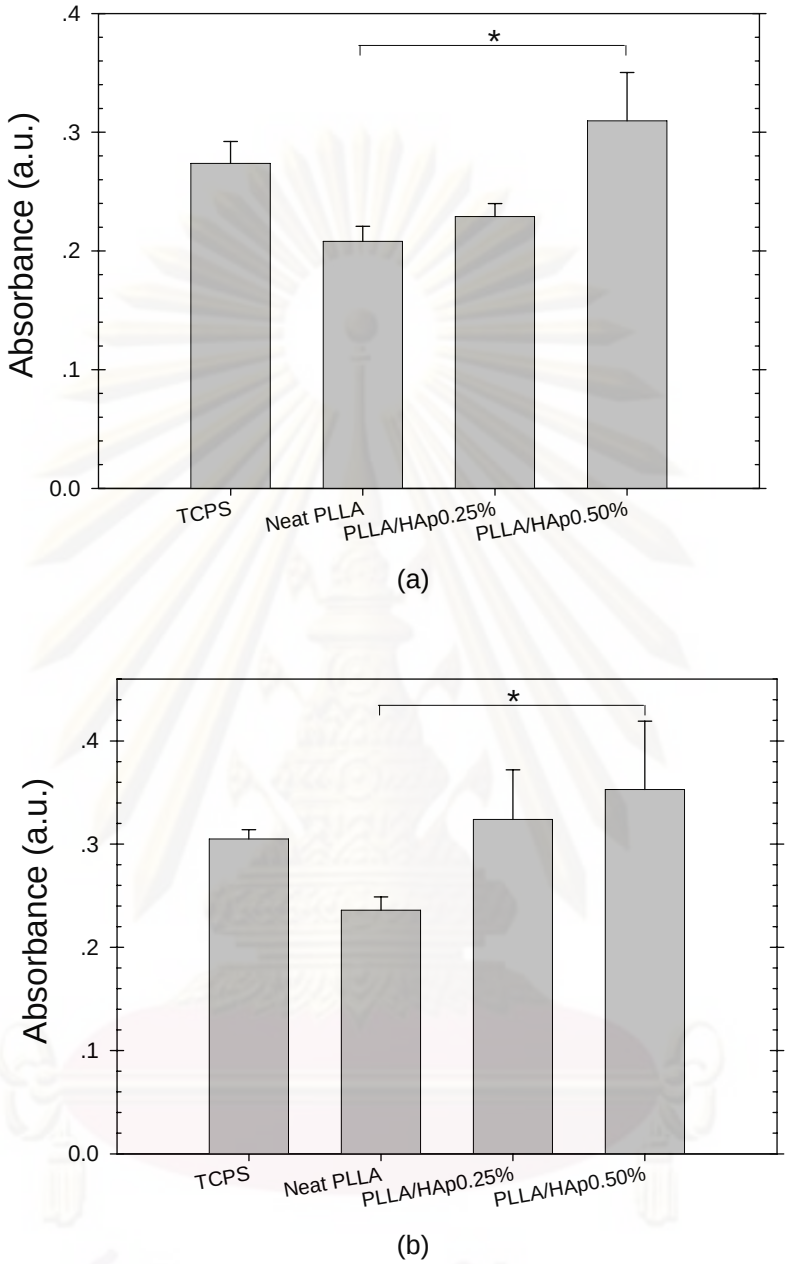


Figure 3.4. (a) Attachment and (b) proliferation of MC3T3-E1 that had been either seeded or cultured for 8 or 24 h on the surfaces of the electrospun PLLA, PLLA/HAp0.25% and PLLA/HAp0.50% fibrous scaffolds and TCPS (positive control). *Significance at $p < 0.05$ between two groups.

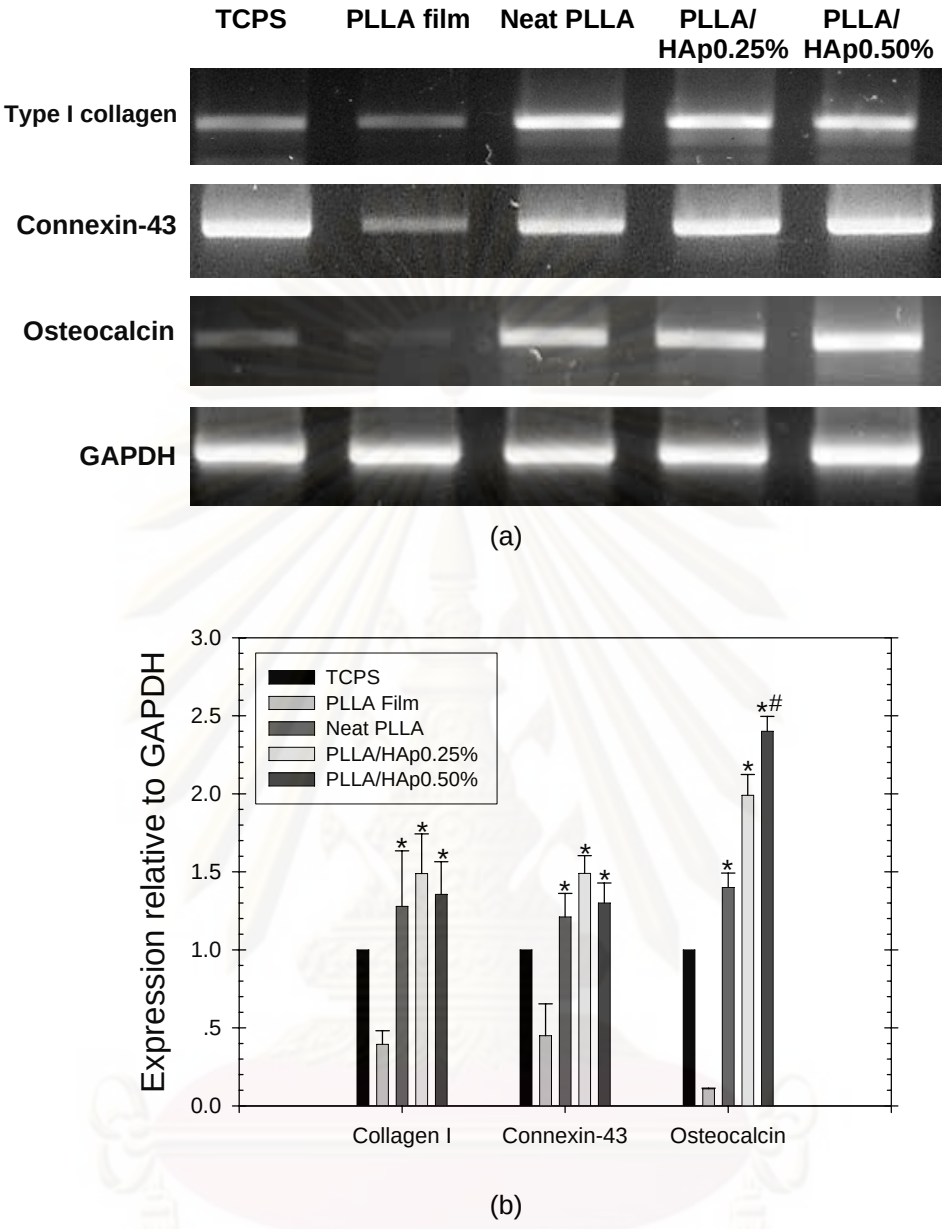
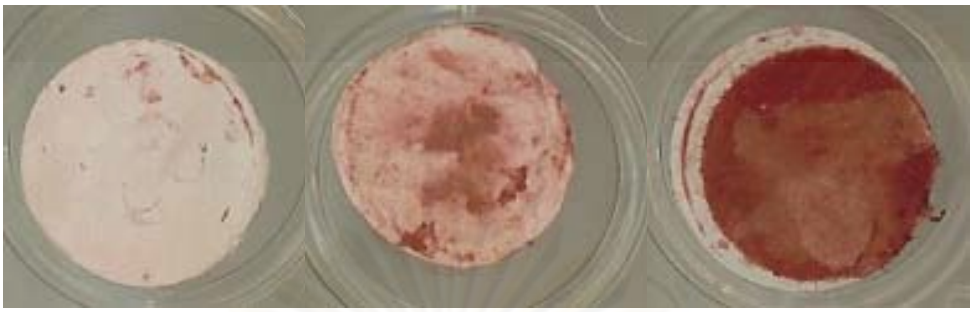
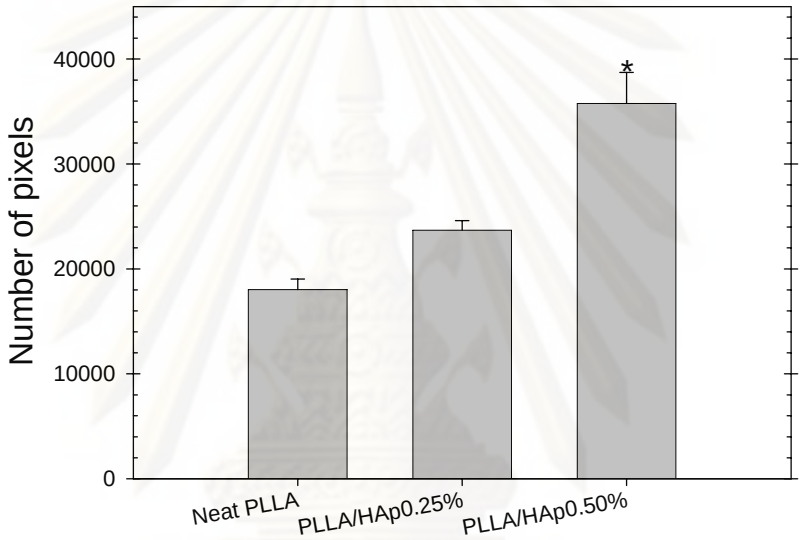


Figure 3.5. Expression of type-I collagen, connexin-43 and osteocalcin mRNAs in MC3T3-E1 after having been cultured on the surfaces of the electrospun PLLA, PLLA/HAp0.25% and PLLA/HAp0.50% fibrous scaffolds as well as on solvent-cast PLLA film (internal control) and TCPS (positive control) for 14 d. Intensities of the bands were either (a) semiquantified or (b) normalized with those of GAPDH and the relative intensities of the respective genes for the cells that had been cultured on TCPS. *Significance at $p < 0.05$ with respect to the PLLA film. $^{\#}$Significance at $p < 0.05$ with respect to the PLLA fibrous scaffold.



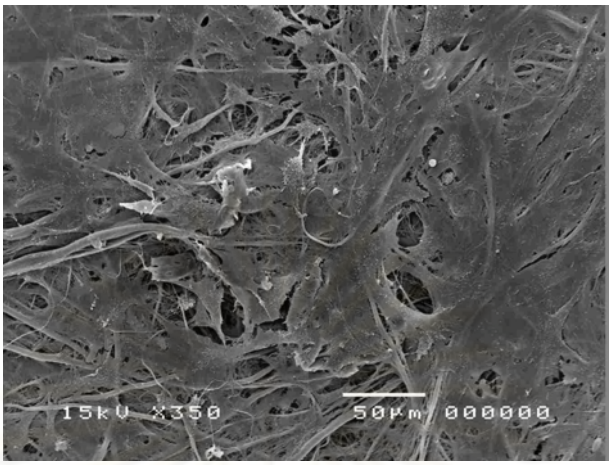
Neat PLLA PLLA/HAp0.25% PLLA/HAp0.50%

(a)

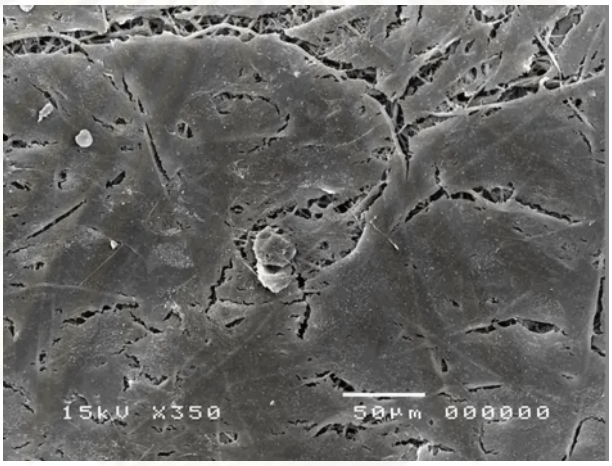


(b)

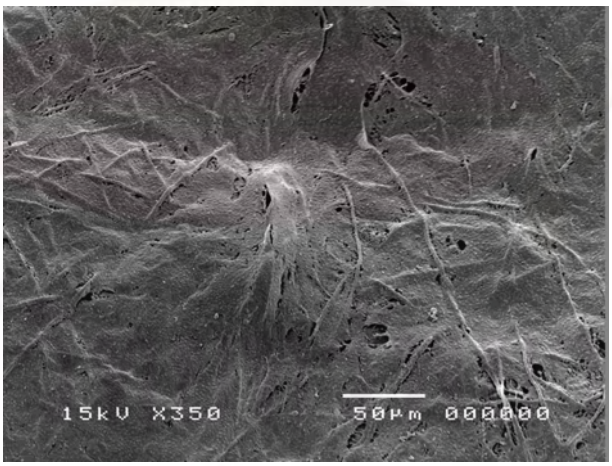
Figure 3.6. Alizarin Red S staining for mineralization assessment of MC3T3-E1 after have been cultured on the surfaces of the electrospun PLLA, PLLA/HAp0.25% and PLLA/HAp0.50% fibrous scaffolds for 21 d: (a) representative photographic images of stained specimens and (b) corresponding analysis for the observed number of red pixels. *Significance at $p < 0.05$ with respect to the PLLA fibrous scaffold.



(a)



(b)



(c)

Figure 3.7. Selected SEM images of cultured fibrous scaffolding specimens, i.e., the electrospun (a) PLLA, (b) PLLA/HAp0.25% and (c) PLLA/HAp0.50% fibrous scaffolds, on day 21 after MC3T3-E1 had been cultured on their surfaces.

Further Discussion

Despite the inherent biodegradability and biocompatibility of PLLA, actual utilization of this material as synthetic prostheses, implants and/or tissue engineering matrices is hampered by its inherent hydrophobicity (Badami et al., 2006), which may lead to foreign body reactions, such as inflammation, infections, aseptic loosening, local tissue waste and implant encapsulation, *in vivo*. Approaches to further improving the biocompatibility of PLLA and similar materials include reduction of unspecific protein adsorption (i.e., non-fouling properties), enhancement of specific protein adsorption and chemical modification by immobilization of certain cell recognition motives on the surface of a substrate to obtain controlled interaction between the cells and the synthetic substrate (Hersel et al., 2003). The enhancement of the adsorption of specific proteins on the surface of a substrate could be done by the addition of some functional substances in the substrate matrix. As an effectual example, specific adsorption of fibronectin and/or osteocalcin on hydroxyapatite (HAp) particles that had been incorporated in the electrospun PCL fibrous scaffolds was postulated as the reason for the ability of the materials to enhance the proliferation and the differentiation of MC3T3-E1 (Wutticharoenmongkol et al., 2007).

Park et al., 2006 utilized the chemical modification pathway to impart the hydrophilicity to the surface of the electrospun PLLA fibrous scaffold. First, the fibrous scaffold was treated with oxygen plasma and acrylic acid (AA) was subsequently graft-copolymerized onto the surface of the oxygenated fibrous scaffold. The ability of the AA-grafted PLLA fibrous scaffold in supporting the proliferation of NIH-3T3 fibroblasts was obviously better than that of the untreated fibrous scaffold and the corresponding film. Enhancement in the osteoconductivity of a bone scaffold has been achieved with the incorporation of HAp particles (Habibovic and Groot, 2007) which could be a result of its specificity for the adsorption of certain ECM proteins, e.g., fibronectin and osteocalcin (Hutmacher et al., 2000, Wutticharoenmongkol et al., 2007). Functionalization of the electrospun PLLA fibers with HAp could be as simple as incorporating HAp particles in the PLLA spinning solution prior to the electrospinning (Kim et al., 2006; Sui et al., 2007; Jeong et al, 2008) or subsequent deposition of HAp particles on the fiber surface (Chen et al., 2006). Kim et al., 2006 showed that incorporation of HAp particles (~35 nm on average) was responsible for the enhancement in the attachment (i.e., at 6 h after cell

seeding) and the early proliferation (i.e., on day 2 after cell culturing) of MG-63 osteosarcoma cells as well as a significant increase in the ALP activity of the cells that had been cultured on the PLLA/HAp fibrous scaffold in comparison with the neat PLLA counterpart for 7 d, despite the equivalent viabilities of the cultured cells on both substrates on day 7. Similarly, Deng et al., 2007 and Sui et al., 2007 found that MG-63 adhered much better on the HAp-incorporated PLLA fiber mat than on the neat fibrous material. Jeong et al., 2008 showed that incorporation of HAp particles (~35 nm) in the electrospun PLLA fibrous matrices enhanced the attachment (based on immuno-staining of cytoskeleton) and the proliferation (based on double-strand DNA content) of MC3T3-E1 pre-osteoblasts.

Here, HAp particles were also incorporated in the electrospun PLLA fiber mats with an aim of improving the osteoconductivity of the resulting PLLA/HAp fibrous matrices over that of the neat PLLA counterparts. Obviously, the incorporation of HAp in the electrospun PLLA fiber mats improved the wettability of their surfaces, as evidenced by the observed decrease in the static water contact angle values. The potential for use of the neat PLLA fiber mats as scaffolding materials for cell/tissue culture was first assessed with a direct culture with L929 mouse fibroblasts, in which the viability of the cells that had been cultured on the fiber mats for 24 and 48 h was greater than that on the films (see Figure 3.3). Among the various fibrous scaffolds, the PLLA/HAp0.50% ones was better than the PLLA/HAp0.25% and, particularly, the PLLA fibrous scaffolds in supporting both the attachment and the proliferation of MC3T3-E1 (see Figure 3.4). The potential for use of the PLLA/HAp fiber mats as scaffolding materials for bone cell culture was further evaluated by focusing on the ability of the fibrous scaffolds in supporting the full osteogenic differentiation of MC3T3-E1. In this regard, the expression of type-I collage, connexin-43 and osteocalcin mRNAs in MC3T3-E1 that had been cultured on the PLLA/HAp fibrous scaffolds and other substrates for comparison purposes for 14 d was investigated. Specifically, type-I collage, the major component of the organic ECM of the bone (i.e., >86%), is expressed early on in the matrix synthesis stage of the osteoblastic differentiation (Hernández-Gil et al., 2006). Connexin-43, a gap junction protein that is responsible for the intercellular communication among adjacent osteoblasts, is expressed on the cytoplasmic processes of the cells (Hauschka et al., 1989; Civitelli et al., 1993). Lastly, osteocalcin, a small ECM protein synthesized specifically by

osteoblasts and platelets, constitutes ~15% of the non-collagenous ECM proteins (Hernández-Gil et al., 2006). Osteocalcin plays an important role in biomineralization by its ability to bind calcium and HAp (Hauschka et al., 1989; Hernández-Gil et al., 2006) and is often regarded as a terminal osteoblastic differentiation marker (Hauschka et al., 1989; Murugan and Ramakrishna, 2006; Wutticharoenmongkol et al., 2006).

According to the results shown in Figure 3.5, the amounts of the mRNAs associated with the expression of type-I collage, connexin-43 and osteocalcin in the cells that had been grown on the neat PLLA fibrous scaffold for 14 d were significantly greater than those in the cells that had been grown on the film counterpart. It is expected that the increase in the porosity, surface area, total adsorption of specific proteins, and/or the ECM-like topography of the fibrous scaffolds may have contributed to this finding (Kim et al., 1998). Among the various fibrous scaffolds, the significant increase in the osteocalcin mRNA level in the cells that had been grown on the PLLA/HAp0.50% fibrous scaffold indicated that the presence of HAp particles in the composite fibrous scaffold plays a role in mediating MC3T3-E1 to differentiate from their pre-osteoblastic phenotype into the full osteoblastic lineage. This finding was accented by the greatest extent of mineralization observed for this particular specimen that had been cultured with the cells for 21 d (see Figure 3.6). A similar finding was also observed for the cells that had been grown on the electrospun PCL/HAp fibrous scaffolds (Wutticharoenmongkol et al., 2007). On the other hand, the amounts of type-I collage and connexin-43 mRNAs that were expressed in the cells that had been cultured on the fibrous scaffolds for various time intervals (i.e., 2-14 d) were not significantly different. Since these two proteins are expressed early on in the matrix synthesis stage (Hauschka et al., 1989; Civitelli et al., 1993; Hernández-Gil et al., 2006) the expression of their mRNAs should also be expressed at the very early stage of the osteoblastic differentiation.

Conclusion

Electrospinning was used to fabricate fiber mats of poly(L-lactic acid) (PLLA; M_n = 200,000 g·mol^{-1}) with or without the presence of HAp particles. The amount of HAp in the composite fibrous materials was either 0.25 or 0.50% (w/v, based on the volume of the base PLLA solution, i.e., 15% w/v in 70:30 v/v dichloromethane/tetrahydrofuran). The average diameters of the neat PLLA, the PLLA/HAp0.25% and PLLA/HAp0.50% fibers were 3.5, 2.3 and 2.5 μm, respectively. These respectively corresponded to the inter-fiber spacing values of 8.5, 6.1 and 5.7 μm on average. The porosity of these fibrous membranes was high (i.e., ~97-98%). The direct cytotoxicity evaluation of the neat PLLA fiber mat with L929 mouse fibroblasts indicated that the fibrous material was non-toxic. The neat PLLA and the PLLA/HAp fiber mats as scaffolding materials for bone cell culture was further evaluated with MC3T3-E1 mouse pre-osteoblastic cells and the results showed that the PLLA/HAp0.50% fibrous scaffold was the best to support both the attachment and the proliferation of the cells. MC3T3-E1 having been cultured on the surfaces of all of the fibrous scaffolds for 14 d expressed greater amounts of osteocalcin mRNA than they did on the neat PLLA film, and, among the fibrous scaffolds, the one containing the greatest amount of HAp particles (i.e., PLLA/HAp0.50%) also showed the greatest amount of osteocalcin mRNA. Along with greatest amount of mineralization observed for the cells that had been grown on the PLLA/HAp0.50% fibrous scaffold for 21 d, the presence of HAp in the PLLA fibrous scaffolds certainly enhanced the osteoconductivity of the materials.

CHAPTER IV
POLYCAPROLACTONE/HYDROXYAPTITE COMPOSITE SCAFFOLDS : PREPARATION, CHARACTERIZATION AND *IN VITRO* AND *IN VIVO* BIOLOGICAL RESPONSES OF HUMAN PRIMARY BONE CELLS

Summary

Background: Polycaprolactone (PCL) is a synthetic biodegradable polymer that has been approved for use as bone graft substitutes. In this study, PCL scaffolds incorporating hydroxyapatite (HAp) particles were fabricated by combined solvent casting and particulate leaching techniques. The average pore dimension was in the range of about 480-500 μm. The porosity, water absorption and compressive modulus of the scaffold were evaluated. The responses of primary bone cells cultured on the PCL and PCL/HAp scaffolds were examined both *in vitro* and *in vivo*.

Results: In comparison with the cells grown on the PCL scaffold, those cultured on the PCL/HAp counterpart positively expressed the markers of osteogenic differentiation. Cells increased the mRNA expressions of type I collagen and osteocalcin on day 10 and demonstrated a significant increase in calcium deposition. In coherence with the *in vitro* appearance, histomorphometric analysis in a mouse calvarial model showed a significantly greater amount of new bone formation.

Conclusion: The results demonstrated that the prepared PCL/HAp scaffold could be a good candidate as synthetic substitute for bone tissue engineering.

Introduction

Some of the common practices that are used to repair bony skeletal defects caused by congenital abnormalities, diseases, injuries or traumas are autografts, allografts or synthetic implanting materials. Yet, there persist imperfections in these methods; namely, limited availability of the harvesting sites, the possibility of disease transmission, poor biocompatibility and the risk of prosthetic implantation failure. Therefore, the need for alternative strategies such as tissue engineering approaches is required to improve the treatment and quality of life for all of the patients (Hutmacher et al., 2000; Ciapetti et al., 2003; Kujala et al., 2003; Kim et al., 2008).

The use of a synthetic polymer in bone tissue engineering is an alternative strategy extensively used nowadays. The most commonly used synthetic polymers for this purpose are polycaprolactone (PCL), polyglycolide (PGA), polylactide (PLA) and their corresponding copolymers. PCL, a US food and drug administration (FDA)-approved material for craniofacial indications, is a biocompatible and biodegradable aliphatic polyester, having a low melting point. PCL dissolves well in common organic solvents, hence it is a prime candidate to be used in bone scaffolding applications (Yeo et al., 2008). Both mechanical and biological (in terms of osteoconductivity and osteoinductivity) properties of a PCL scaffold can be improved by the addition of bioactive materials. Hydroxyapatite (HAp: $Ca_{10}(PO_4)_6(OH)_2$) is a bioceramic which has the same chemical composition (Ca/P = 1.67) as that of the bone mineral.

Despite its inherent osteoconductivity, HAp has some undesirable traits, such as brittleness and difficulty of shaping. These limit its practicality as bone substitutes. As a result, the fabrication of a biodegradable polymer in combination with the bioceramic could be a solution to such problems (Kim et al., 2006). We have shown previously that electrospun PCL/HAp fibrous matrices could support much better cell attachment, proliferation, differentiation and *in vitro* mineralization of human osteosarcoma cells (SaOS-2) and mouse calvaria-derived pre-osteoblasts (MC3T3-E1) than did the PCL and the PCL/calcium carbonate ($CaCO_3$) counterparts, indicating that PCL/HAp could be a suitable material for bone scaffolding (Wutticharoenmongkol et al., 2006, 2007).

Furthermore, another major factor that determines the success of a bone scaffold is the optimal pore size and porosity, which can be tailored by the variation in both the size and the content of the porogen particles used. The development of the porous PCL/HAp scaffolds was shown in the present contribution in a step by step manner by the combined solvent casting and particulate leaching techniques. The effects of the PCL to porogen ratios, the presence of HAp particles and hydrolytic treatment with alkaline solutions on the morphology, mechanical integrity and the physico-chemical properties of the porous scaffolds were carefully investigated. The potential for use of the PCL/HAp scaffolds as bone scaffolding materials was assessed *in vitro* in terms of the ability of the composite scaffolds to support the proliferation, the expression of genes of certain bone marker proteins and the mineralization of the cultured primary bone cells. Finally, the scaffolds were assessed in vivo based on a calvarial defect model in mice.

Experimental details

Materials and preparation of PCL and PCL/HAp scaffolds

Polycaprolactone (PCL; Aldrich, USA; M_w = 80,000 g·mol^{-1}) scaffolds were fabricated by the combined solvent casting and particulate leaching techniques. Chloroform (Labscan (Asia), Thailand) and sucrose (Fluka Chemika, Switzerland) were used as the solvent and the porogen, respectively. Hydroxyapatite powder (HAp) was synthesized following the method proposed by Shih et al., 2004. The mean size of the as-synthesized HAp powder was 234 ± 68 nm.[7-9] PCL was first dissolved in chloroform with varying amounts of HAp (0-50% w/w of PCL) being added later. Sucrose, sieved into 400-500 μm particles, was added to the PCL/HAp suspensions. Unless otherwise noted, the ratio of PCL to sucrose was 1:10 w/w. The mixture was poured into a mould of a cylindrical cavity (14 mm in height and 14 mm in diameter) and allowed to dry for 24 h. The scaffolds were immersed in distilled water for 2 d to dissolve away the porogen (with the water being replaced in every 4 h) and later dried *in vacuo* for 48 h prior to further use. To improve the hydrophilicity, scaffolds were immersed in 1 M sodium hydroxide (NaOH; Ajax Finechem, Australia) aqueous solution at 37 °C for 6 h. The alkaline-treated scaffolds were washed thoroughly in distilled water and dried *in vacuo* for 48 h. (Pena et al., 2006). For sterilization, the scaffolds were placed in 70% v/v ethanol for 30 min and washed with sterilized de-ionized water.

Characterization of PCL and/or PCL/HAp scaffolds

Morphology and pore dimensions

The porous scaffolds were cut with a razor blade into two halves at the center of their height and processed for scanning electron microscopic (SEM) analysis. The scaffolds were coated with gold using a JEOL JFC-1100E sputtering device and examined with a JEOL JSM-5410LV scanning electron microscope. The pore dimensions of the neat PCL scaffolds were determined from the obtained SEM images. For a given pore, both the width and the height were recorded. These values were averaged to obtain the pore dimension of the particular pore. More than 30 pores for each sample group were measured.

Bulk density and porosity values

The bulk densities of the porous scaffolds were determined using a liquid displacement method (Zhang et al., 1999). Specifically, each scaffold specimen (of which the initial dry weight, W, was recorded) was immersed in a graduated cylinder containing a known volume (V_1) of ethanol. The cylinder was placed *in vacuo* to drive ethanol to infiltrate into the pore structure of the scaffold. The total volume of the remaining ethanol and the ethanol-infiltrated scaffold were then recorded as V_2 by reading the level of the graduated cylinder. The ethanolinfiltrated scaffold was then removed from the graduated cylinder and the residual volume of ethanol was recorded as V_3. Based on these data, the bulk volume of the scaffold can be calculated as V_2-V_3; the pore volume as V_1-V_3; the bulk density of the scaffolds as $\rho_s = W/(V_2 - V_3)$; and, finally, the porosity of the scaffold as $(V_1-V_3)/(V_2-V_3)\times 100\%$.

Mechanical properties

The compressive moduli of the porous scaffolds were investigated with a Lloyd LRX universal testing machine using a 450 N load cell and a crosshead speed of 2 $mm \cdot min^{-1}$. The load was applied on the top of each scaffold specimen until it was compressed by about 75% of its original height. The compressive modulus was represented as the slope of the linear portion of the stress-strain curve over the compressive strain range of 10-20%.

Actual content of HAp

The actual contents of HAp within the porous PCL/HAp scaffolds were determined by a Perkin-Elmer Pyris Diamond thermogravimetric/differential thermal analyzer (TG/DTA). A specimen weighing about 20 ± 2 mg from each sample group was randomly selected. Each specimen was heated from 30 ºC at a heating rate of 20 $ºC \cdot min^{-1}$ to 900 ºC. DTA results were used to provide information about the decomposition temperatures of the scaffolds, while TGA results were used to provide the actual HAp contents.

Water retention

The dry scaffold specimens were weighed and then placed in a glass bottle filled with 25 mL of distilled water for 1, 3 and 5 min. Upon removal, the scaffolds

were carefully wiped with a piece of filter paper to remove excess water on their surface, after which the wet weights of the specimens were recorded. The amounts of water retained (i.e., water retention) within the specimens were calculated from their initial, dry weights (W_d) and measured wet weights (W_w): i.e., $(W_w-W_d)/W_d$ x100%. Note that the amounts of water retained within the scaffolds include the amounts of water absorbed by the mass of the scaffolds and those retained within the pore structure (Shih et al., 2004).

Cell culture

Primary human bone cells were obtained form alveoloplasty, torus palatinus or torus mandibularis removal for prosthodontic reasons as previously described (Coombes et al., 2004; El-Amin et al., 2006). All patients gave informed consent. The protocol has been approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. All bone chips were washed extensively in sterile phosphate buffer saline (PBS, pH 7.4), cut into smaller pieces and digested with 0.25% trypsin-EDTA to remove residual adipose and hematopoietic tissues. The bone chips were harvested in 35 mm culture dish (Nunc, USA) and grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, USA), containing 15% fetal bovine serum (FBS; ICP biologicals, New Zealand), 2mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 5 μg/mL amphotericin B (GIBCO, USA) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After human primary bone cells reached the confluence, cells were sub-cultured at a 1:3 ratio. Cells were stained for alkaline phosphatase (ALP) activity, using an ALP staining kit (TAKARA Bio, Japan) to confirm the primary characteristic of the bone cells. The cell passages from 3 to 6 were used in this study. All experiments were performed in triplicate using cells prepared from three different donors.

Cell Proliferation

Cell proliferation was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; USB Corporation, USA) assay. This assay is based on the ability of viable cells to reduce a tetrazolium-based compound, MTT, to a purplish formazan product. In brief, cells were seeded onto a scaffold specimen at a density of 50,000 cells/well. At the end of the culture period, the medium was replaced with

MTT solution for 30 min at 37 ºC and the formazan product was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich, Germany). The absorbance of the supernatant was evaluated with a Thermospectronic Genesis 10 UV-visible spectrophotometer at 570 nm.

Reverse-transcription Polymerase Chain Reaction (RT-PCR)

Cells were seeded on tissue-culture plate (TCP), PCL and PCL/HAp scaffold specimens at a density of 120,000 cells/well in a 12-well TCP for 2, 5 and 10 d. Total cellular RNA was extracted with Tri reagent (Roche Diagnostics, USA) according to the manufacturer's instructions. Exactly 1 µg of each RNA sample was converted to cDNA by avian myeloblastosis virus (AMV) reverse transcriptase (Promega, WI) for 1.5 h at 42 ºC. Subsequently, a polymerase-chain reaction (PCR) was performed. The primers were prepared following the reported sequences from GenBank. The oligonucleotide sequences of the primers are type I collagen forward 5' CTG GCA AAG AAG GCG GCA AA 3', reverse 5' CTC ACC ACG ATC ACC ACT CT 3', osteocalcin forward 5' ATG AGA GCC CTC ACA CTC CTC 3', reverse 5' GCC GTA GAA GCG CCG ATA GGC 3' and GAPDH forward 5' TGA AGG TCG GAG TCA ACG GAT 3', and reverse 5' TCA CAC CCA TGA CGA ACA TGG 3'.

Semi-quantitative PCR was performed using Tag polymerase (Tag DNA Polymerase, Invitrogen, Brazil) with a reaction volume of 25 µL containing 25 pmol of primers and 1 µL of RT product. The amplification profile was 1 cycle at 94 °C for 1 min, 28 cycles at 94 °C for 1 min, hybridization at 60 °C for 1 min and extension at 72 °C for 2 min for Type I collagen and osteocalcin (22 cycles for GAPDH), followed by 1 extension cycle of 10 min at 72 °C. The PCR was performed in the DNA thermal cycler (Biometra GmH, Germany). The amplified DNA was then electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide (EtBr; Bio-Rad, USA) fluorostaining. All bands were scanned and analyzed using Scion image analysis software.

Alizarin Red-S Staining and Calcium Quantification

To examine the bone nodule formation, the cells were seeded at a density of 50,000 cells/well on TCP or the scaffold specimens. After 3 d in culture, the medium was changed to the one that contained an osteogenic supplement, i.e., ascorbic acid

(50 μg/mL; Sigma, USA) and β-glycerophosphate (5 mM; Sigma, USA), and incubated further for another 21 d, with a change of the medium on every other day. Calcium deposition was investigated by Alizarin Red-S staining (Sigma, USA). Scaffolds were fixed with cold methanol for 10 min, washed with deionized water and immersed in 1% Alizarin Red-S solution (Sigma, USA) [in a mixture of 0.4 mL ammonium hydroxide/40 mL water (pH = 4.2)] for 3 min. The amount of calcium was quantified by destaining with 10% cetylpyridinium chloride monohydrate (Sigma, USA) in 10 mM sodium phosphate at room temperature for 15 min and spectrophotometically read at 570 nm.

Mouse calvarial defects and histomorphometric analysis

The experiment was carried out on eight weeks-old male (ICR Mouse) mice (National Laboratory Animal Centre, Mahidol University, Thailand). The protocol was approved by the Animal Care and Use Ethical Committee, Faculty of Medicine, Chulalongkorn University. Two circular calvarial defects (4 mm in diameter) were created under general anesthesia with Avertin by intraperitoneal injection. The wound was closed with a 4-0 nylon suture. A total of 6 mice were used, which translated to 12 calvarial defects in total. Four randomly-selected defects were implanted with PCL scaffolds. Another four defects were implanted with the selected PCL/HAp scaffolds. The rest of the defects were closed with no implantation (i.e., control group). At 6 weeks, the mice were sacrificed and the calvarial bone was carefully excised, cleaned and fixed immediately with 4% formaline (24 h at 4 ºC). The specimens were dehydrated in graded ethanol solutions, embedded in paraffin, sectioned (10 μm in thickness) and stained with Masson's Trichrome. The digital images of the sections were scanned by a visual slide microscope (Mirax desk, Carl Zeiss, Germany). Histomorphometric measurements of each section were done by Scion image analysis program.

Statistical analysis

Statistical analysis of independent *t* tests of triplicate determination was performed. Differences at $p < 0.05$ were considered statistically significant.

Results

Characterization of PCL and/or PCL/HAp scaffolds

Combined solvent casting and particulate leaching techniques were used to fabricate porous scaffolds by using sucrose as the porogen to generate an open-pore structure. The effect of PCL to sucrose ratio on morphological appearance of the neat PCL scaffolds is illustrated in Figure 4.1A. Note that the sizes of sucrose were in the range of 400-500 μm. The pore interconnectivity of the scaffolds increased with an increase in the amount of sucrose, with the scaffold obtained at the PCL to sucrose ratio of 1:15 showing an ill-defined cubical structure. The pore dimensions of the obtained scaffolds ranged between 478 and 502 μm on average ($n \geq 30$), with no particular dependence on the PCL to sucrose ratios. Obviously, the pore dimensions correlated well with the sizes of the porogen used.

The dimensions and the interconnectivity of the pores of the scaffolds affect not only the transport of nutrients into and wastes out of the cells through the pore structure of the scaffolds, but also other properties of the scaffolds. As shown in Figure 4.1B, the porosity increased with an increase in the sucrose loading, with the property values ranging from 81 to 93% on average ($n = 7$). On the other hand, the density values of the porous scaffolds showed a reverse trend, with the property values decreasing from 0.17 to 0.08 $g \cdot cm^{-3}$ on average ($n = 7$). The decrease in the density was in accord with the observed decrease in the rigidity of the porous scaffolds, as the compressive modulus was found to decrease from 0.93 MPa at the PCL to sucrose ratio of 1:5 to 0.05 MPa at the PCL to sucrose ratio of 1:15 ($n = 7$) (results not shown). Among the various PCL scaffolds obtained, the ones that had been prepared at the PCL to sucrose ratio of 1:10 were chosen, based on the balance between the porosity and the morphological integrity, for subsequent studies.

Figure 4.2A showed the porosity and the density values of the PCL/HAp scaffolds that had been prepared at the PCL to sucrose ratio of 1:10 as a function of the initial HAp content (i.e., 0-50% w/w). The presence of HAp increased the density but had no significant effect on the porosity values of the scaffolds. Specifically, the porosity values of the PCL/HAp scaffolds were in the range of 86-88% on average ($n = 7$), with no specific correlation with the initial HAp content. On the contrary, the density value increased rather monotonously with an increase in the initial HAp

content, i.e., from about 0.10 $g \cdot cm^{-3}$ on average for the neat PCL scaffolds and the PCL/HAp scaffolds that had been prepared at the initial HAp content of 10% to about 0.13 $g \cdot cm^{-3}$ on average for the ones that had been prepared at the initial HAp contents of 40 and 50% (n = 7). With regards to the mechanical integrity of the PCL/HAp scaffolds, the presence of HAp obviously improved the compressive rigidity of the scaffolds in an increasing manner with the initial HAp content (see Figure 4.2B). Specifically, the compressive modulus increased from about 0.13 MPa on average for the neat PCL scaffolds to reach the maximum value of about 0.32 MPa on average for the PCL/HAp scaffolds that contained 50% of HAp (n = 10). The PCL/HAp scaffolds that contained 40% w/w of HAp were chosen, based on their greatest porosity values, for subsequent studies.

Prior to further investigation, it is interesting to determine the actual contents of HAp within the obtained PCL/HAp scaffolds. During the leaching of sucrose particles to obtain the porous scaffolds, it is likely that some amounts of HAp particles could also be leached out. The DTA thermograms showed that the presence of HAp particles did not affect certain thermal behavior of the PCL/HAp scaffolds, as the end temperatures of both the melting and the thermal degradation endotherms were observed at about the same temperatures, regardless of the sample types, at 65 and 450 °C, respectively (results not shown). Based on this information, the char contents at 900 °C in the TGA thermograms could be referred to as the actual amounts of the as-loaded HAp particles within the scaffolds. For the PCL/HAp scaffolds that had been prepared with the initial HAp contents of 10, 20, 30, 40 and 50% w/w, the actual amounts of HAp were determined to be 9.8, 16.7, 23.4, 29.2 and 32.1% w/w, respectively. Obviously, the greater the initial HAp content was, the greater the difference between the initial and the actual values would be. The distribution of the HAp particles within the mass of the scaffolds is another important concern. According to Figure 4.2C which illustrates the representative SEM image of the PCL/HAp scaffolds that had been prepared at the initial HAp content of 40%, it is clear that HAp particles distributed rather homogeneously throughout the mass of the PCL matrix. The ability to absorb a great amount of water is a prerequisite of a functional scaffold in actual applications. PCL, due to its relatively hydrophobic nature, requires certain modifications to improve its hydrophillicity. Here, the neat PCL scaffolds that had been prepared at the PCL to sucrose ratio of 1:10 were

immersed in NaOH aqueous solutions of varying concentrations for either 6 or 24 h. The results in Figure 4.3A show that the alkaline treatment enhanced the ability of the scaffolds to absorb water. Clearly, the amount of water retained within the mass and the pore structure of the scaffolds increased with both the concentration of the alkaline solution and the submersion time. Though not shown, the contact angles of sessile water drops on the surfaces of the treated scaffolds decreased with an increase in the NaOH concentration as well.

Figure 4.3B shows the compressive rigidity of the scaffolds after the alkaline treatment. Apparently, immersion of the scaffolds in the NaOH aqueous solutions of varying concentrations for 6 h resulted in a monotonous decrease in the compressive moduli from about 0.13 MPa on average for the untreated scaffolds and the scaffolds that had been immersed in 0.1 M NaOH solution to 0.11 MPa on average for the scaffolds that had been immersed in 4 M NaOH solution (n = 7). Increasing the immersion time to 24 h resulted in a considerable reduction in the compressive moduli. Specifically, the compressive moduli decreased to 0.11 MPa on average for the scaffolds that had been immersed in 0.1 M NaOH solution and finally to 0.05 MPa on average for the scaffolds that had been immersed in 4 M NaOH solution (n = 7). The decrease in the compressive moduli with both the NaOH concentration and the immersion time was in accord with the loss of the integrity of the cellular structure of the scaffolds and the observed increase in the loss of the mass of the scaffolds (results not shown). Based on the obtained results, the scaffolds that had been immersed in 1 M NaOH solution for 6 h were chosen, based on the compromise between the improvement in the water retention behavior and the deterioration in the mechanical integrity, for subsequent studies.

In vitro biological behavior of primary human bone cells on PCL and PCL/HAp Scaffolds

Cell proliferation

The results from the MTT assay showed that the number of primary human bone cells that had been cultured on the PCL/HAp scaffolds was greater than that of the cells that had been cultured on the neat PCL scaffolds at both 24 and 48 h after cell culturing (see Figure 4.4A). Representative SEM images revealed that the cells on

both types of the scaffolds at 24 h after cell culturing expanded well and exhibited the normal morphology (see Figure 4.4B).

Comparison of gene expression by RT-PCR analysis

The expression of type I collagen and osteocalcin mRNAs in the cultured cells was analyzed by RT-PCR at 2, 5 and 10 d of cell culturing on both types of the scaffolds. The results in Figure 4.5A indicate that the expression levels of type I collagen and osteocalcin mRNAs were greater in cells cultured on the PCL/HAp scaffolds than those in cells cultured on the neat PCL scaffolds and TCP on day 10. The graph in Figure 4.5B shows the intensity of the bands shown in Figure 4.5A, which confirms significant increases in both of the type I collagen and the osteocalcin mRNA expression levels in cells cultured on PCL/HAp scaffolds. Notwithstanding, the difference in the expression levels of type I collagen and osteocalcin mRNAs in cells that had been cultured on both types of the scaffolds on days 2 and 5 was not significant (data not shown).

***In vitro* calcification**

Cells were cultured on TCP, the neat PCL and the PCL/HAp scaffolds for 21 d and the *in vitro* calcium deposition was examined by Alizarin Red-S staining (Figure 4.6A). The amount of calcium deposition from Figure 4.6A, quantitated by elution with cetylpyridinium chloride, was shown in Figure 4.6B. The results revealed that the formation of mineralized nodules of the cells cultured on the PCL/HAp scaffolds was significantly greater ($p < 0.01$) than that of the cells cultured on the neat PCL scaffolds and TCP.

***In vivo* bone formation by implantation of PCL and PCL/HAp scaffolds**

The sections of calvaria after 6 weeks of implantation, as shown in Figure 4.7, showed that the calvarial defect of the mice without the implantation (i.e., control) was filled with a thin, loose connective tissue with minimal mineralization. In the defect implanted with a neat PCL scaffold, the scaffold was still intact with some new bone formation and vascularization inside the scaffold. On the other hand, areas of new bone formation were more discernible in the PCL/HAp scaffold. A greater magnification image of the section from the PCL/HAp scaffolding implant showed the

osteoid formation along the edge of the newly-generated bone area. In addition, woven bone structures, osteocytes and lacuna structures were prominent in the implantation (see Figure 4.8A). Histomorphometric analysis indicated an increase in the amount of new bone formation in all of the PCL/HAp scaffolding implants as compared to all of the neat PCL counterparts (see Figure 4.8B).

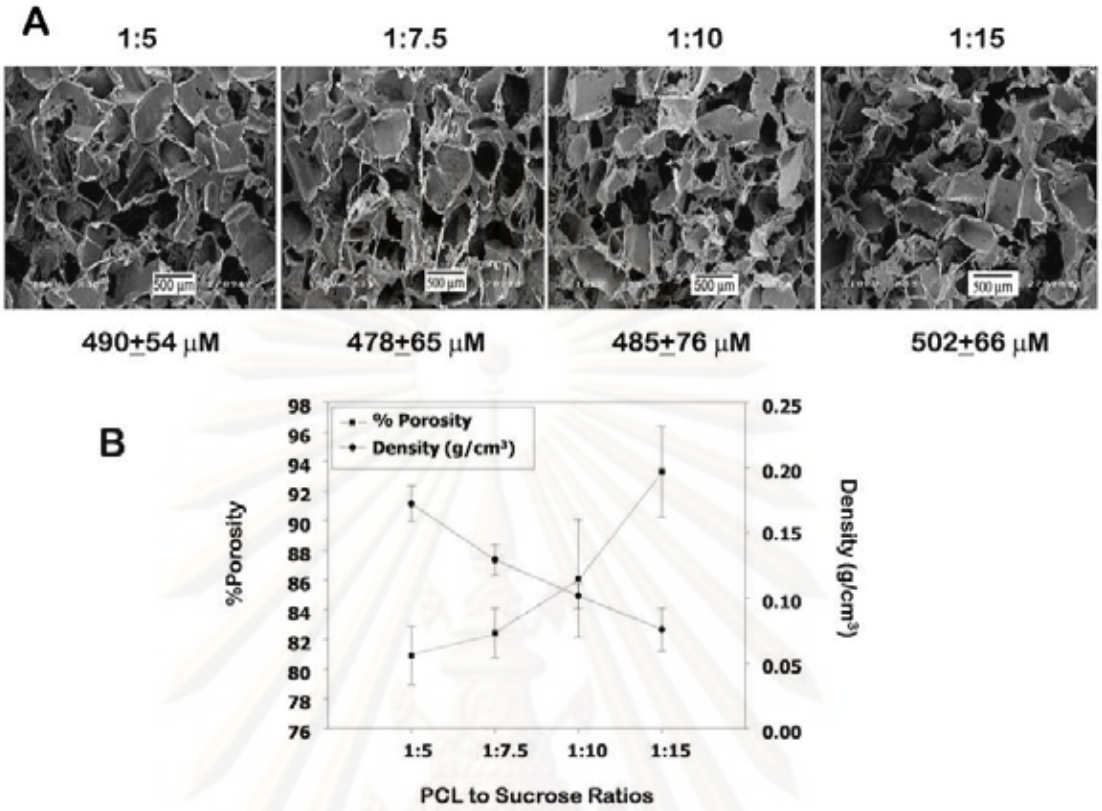


Figure 4.1. (A) Representative SEM images (magnification = 35X and scale bar = 500 µm) illustrating morphology of PCL scaffolds that had been prepared at various PCL to sucrose ratios and the corresponding pore dimension information.

(B) The porosity and the density of the obtained scaffolds at various PCL to sucrose ratios.

(From Wipawan Inrung 2007)

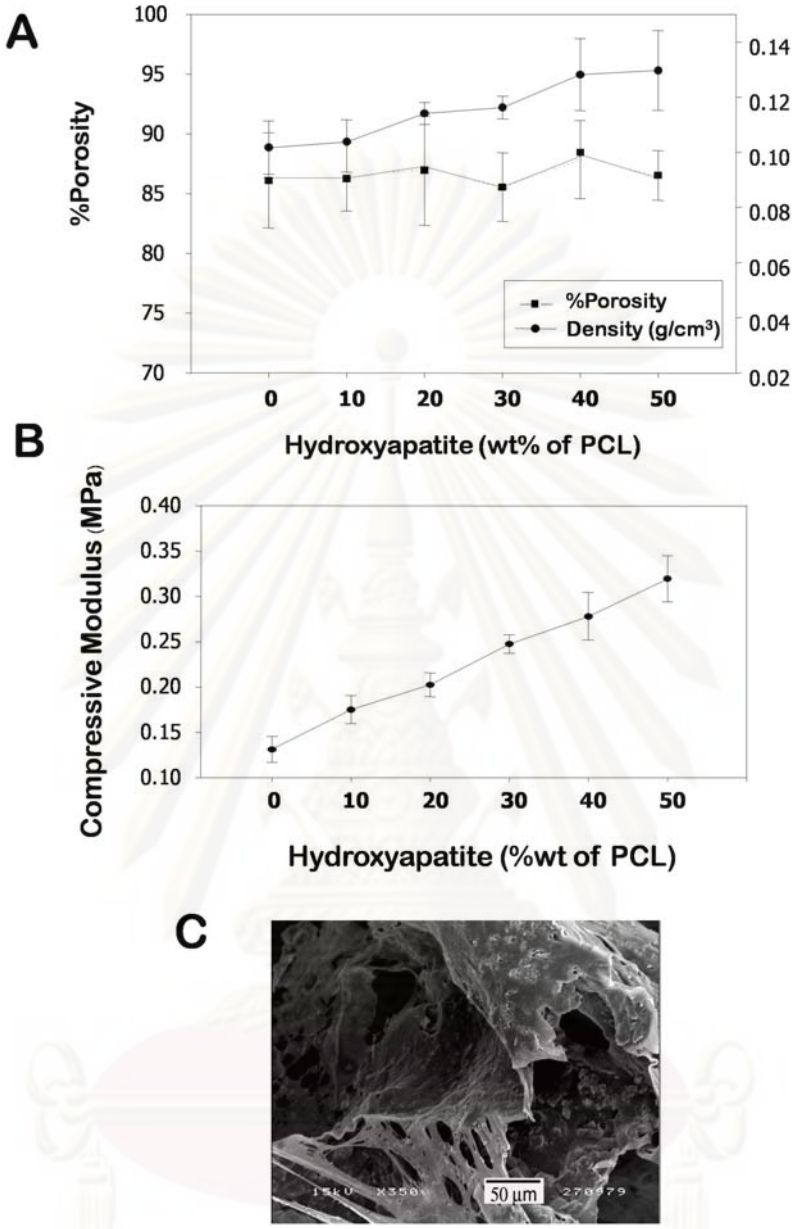


Figure 4.2. (A) Porosity and bulk density values and

(B) compressive moduli of the PCL/HAp scaffolds that had been prepared at the PCL to sucrose ratio of 1:10 w/w as a function of the initial HAp content.

(C) Representative SEM image (magnification = 350X and scale bar = 50 µm) illustrating the morphology of selected cellular compartments of a PCL/HAp scaffold that had been prepared at the PCL to sucrose ratio of 1:10 w/w and the initial HAp content of 40% w/w.

(From Wipawan Inrung 2007)

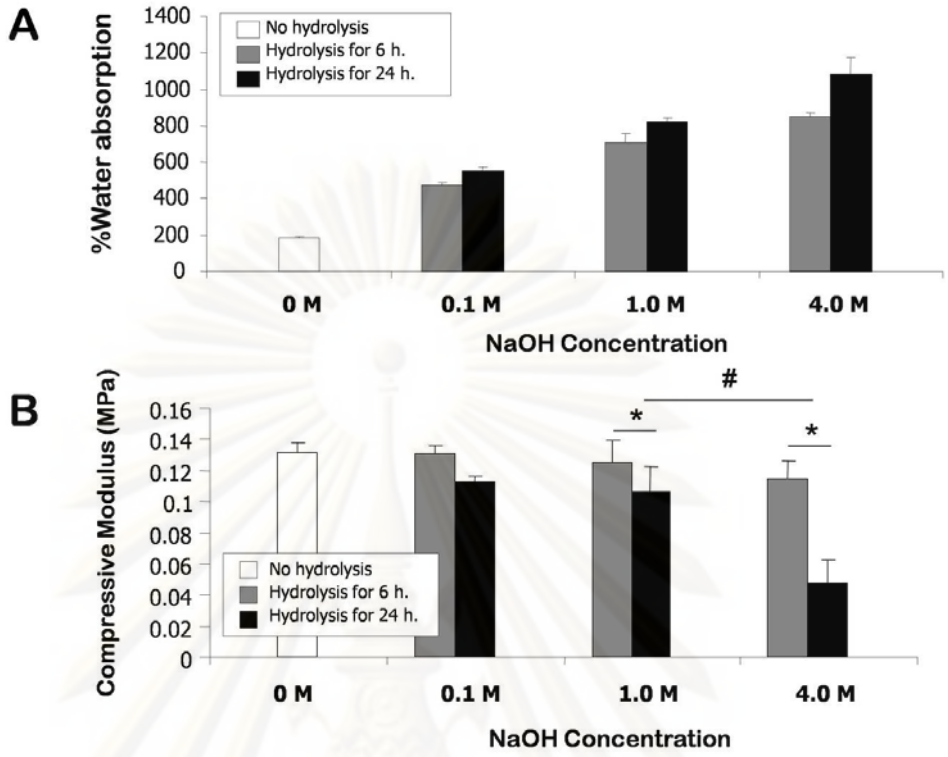


Figure 4.3. (A) Water absorption values and

(B) compressive moduli of PCL scaffolds that had been prepared at the PCL to sucrose ratio of 1:10 w/w after immersion in NaOH aqueous solutions of varying concentrations for ether 6 h (gray bars) or 24 h (black bars).

*, # Significance at $p \leq 0.05$ between the pairs of sample groups as indicated by thick lines.

(From Wipawan Inrung 2007)

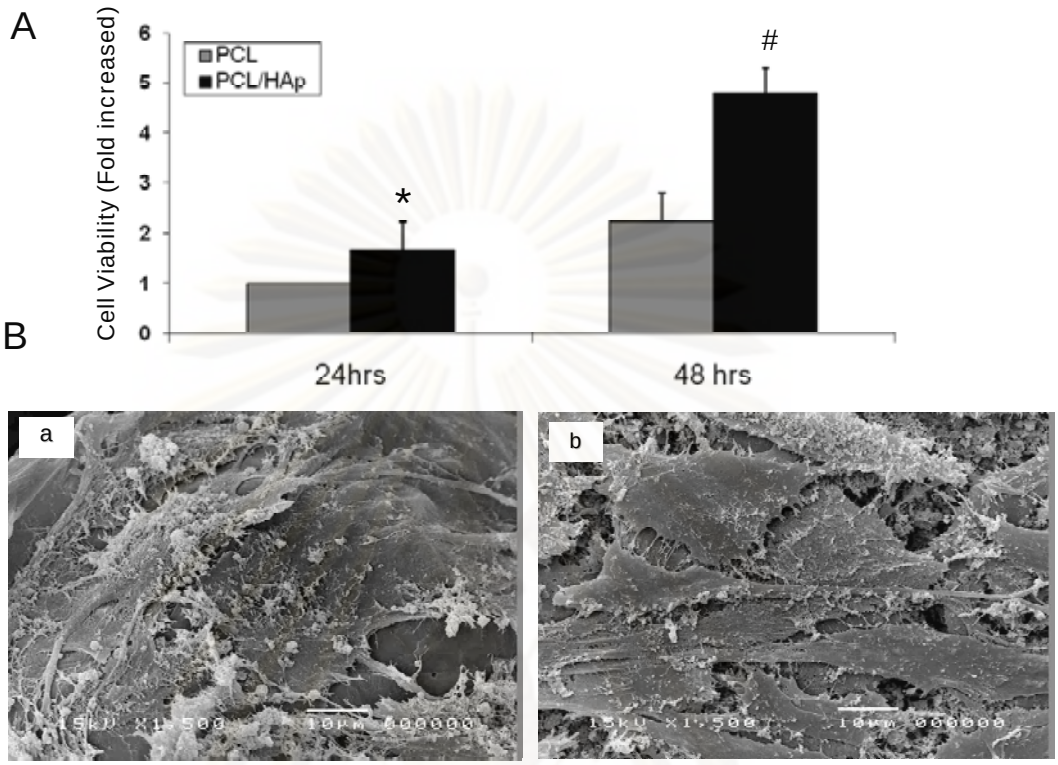


Figure 4.4. (A) Primary bone cells viability that had been cultured on PCL and PCL/HAp scaffolds for 24 and 48 h. These values were determined by MTT assay and were reported relative to the value obtained for the PCL scaffolds at 24 h.

*, # Significance at $p \leq 0.05$ and 0.01, respectively.

(B) Representative SEM images (magnification = 1500X and scale bar = 10 μm) illustrating the morphology of primary bone cells that had been cultured on (a) PCL and (b) PCL/HAp scaffolds for 24 h.

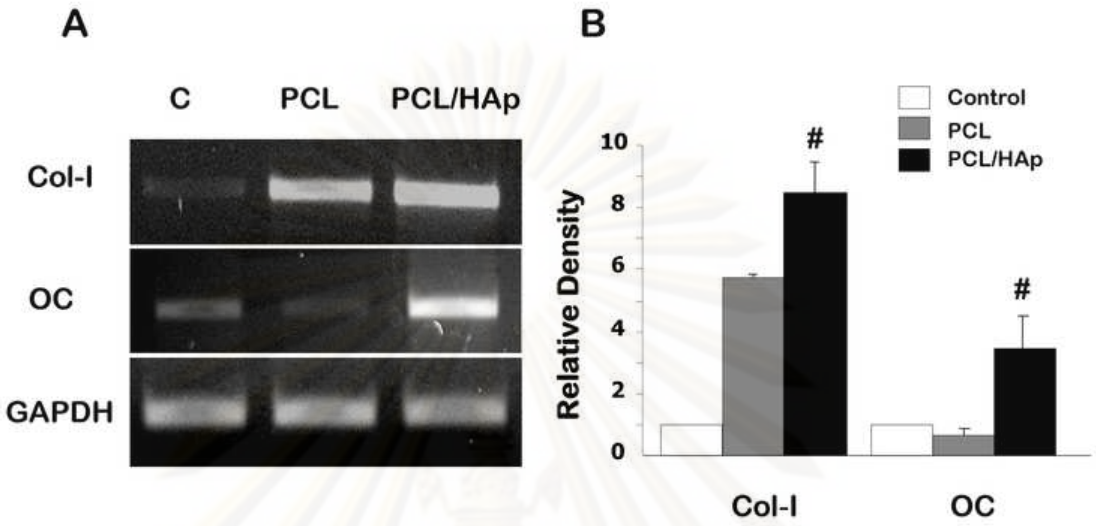


Figure 4.5. (A) Expression of type-I collagen (Col-I) and osteocalcin (OC) mRNAs in primary bone cells that had been cultured on PCL and PCL/HAp scaffolds for 10 d. The results showed increased expression levels of Col-I gene in cells cultured on both types of scaffolds and of OC gene in cells cultured on PCL/HAp scaffolds.

(B) The band intensities of PCR products shown in (A) were quantitated and normalized with those of glyceraldehydes 3-phosphate dehydrogenase (GAPDH), the house keeping gene used as an internal control, and are presented as means ± SD relative to the control (C) from three independent experiments. # Significance at $p \leq 0.01$.

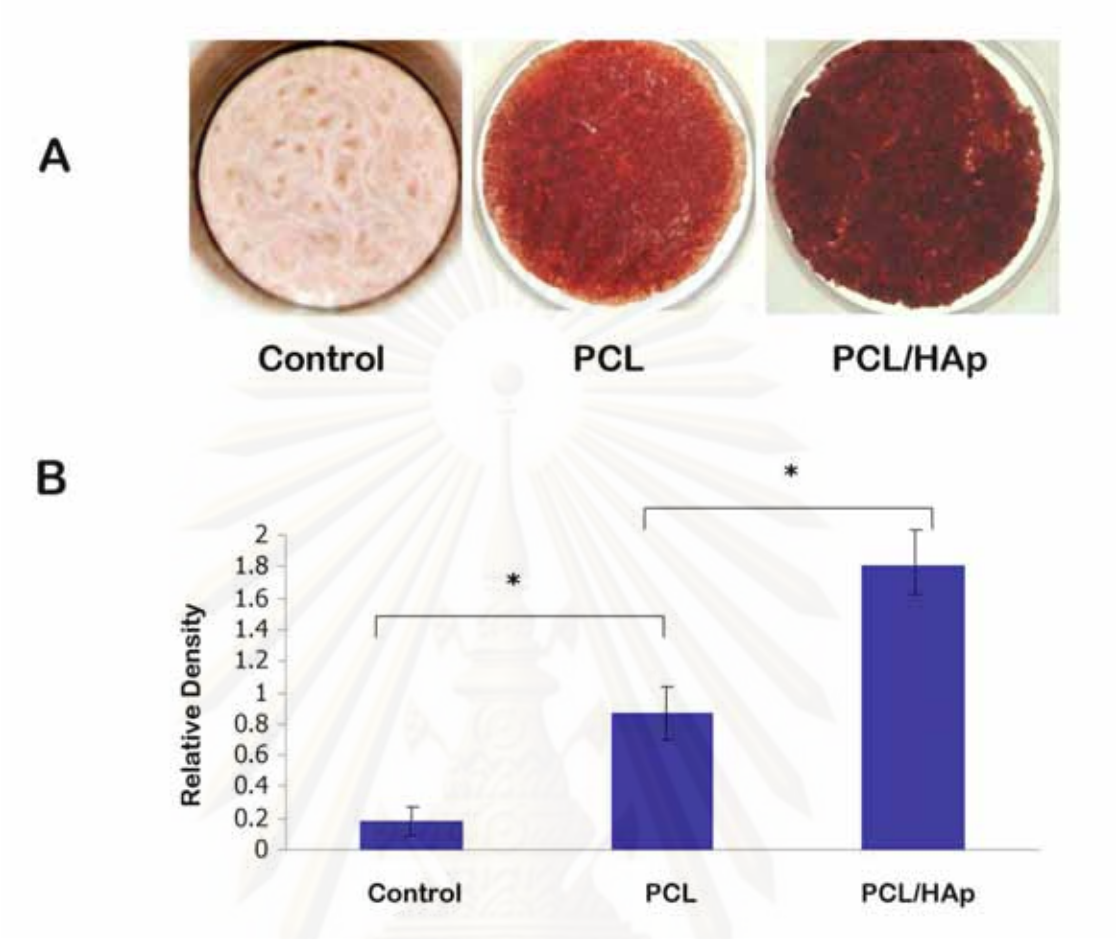


Figure 4.6. (A) Photographic images illustrating Alizarin red S staining for mineralization assessment of cellular constructs after primary bone cells had been cultured on culture plate (Control) and PCL and PCL/HAp scaffolds for 21 d.

(B) Quantification of calcium deposition by elution with cetylpyridinium chloride and spectrophotometically read at 570 nm. # Significance at $p \leq 0.01$.

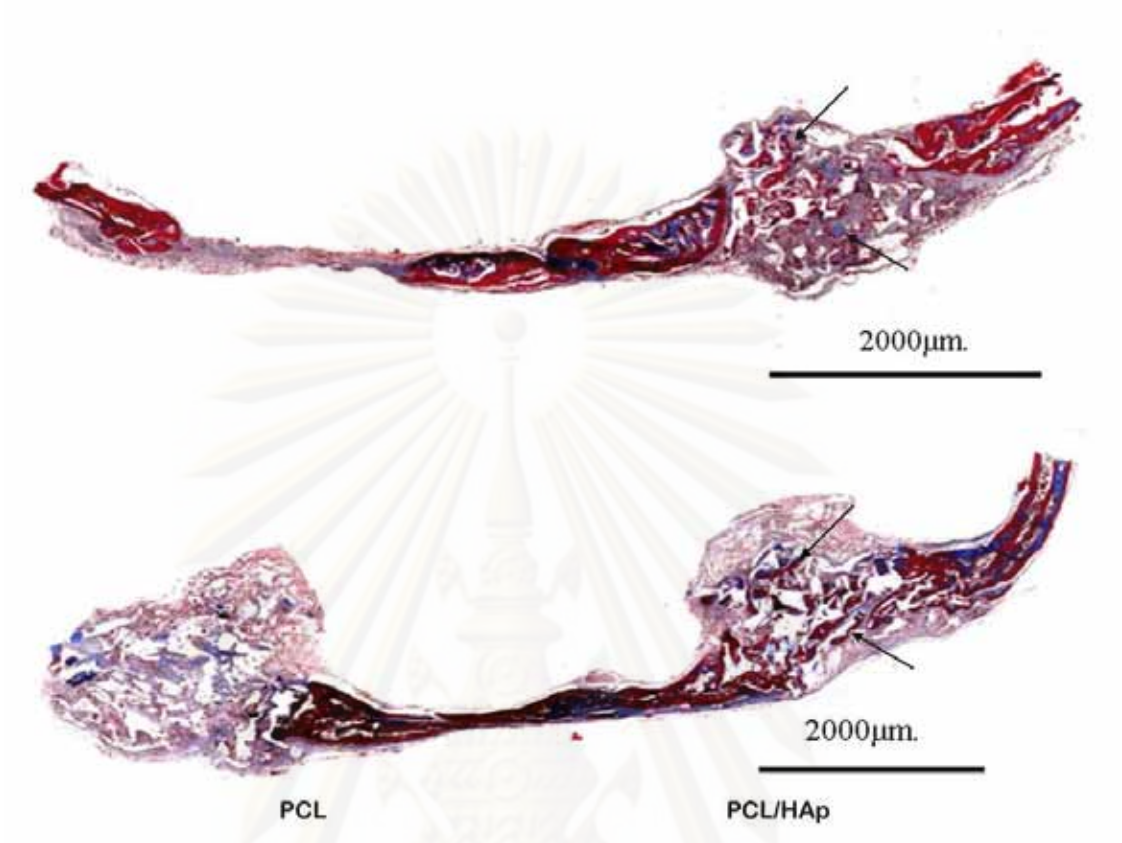


Figure 4.7. Histological evaluation of the calvaria defects at 6 weeks after implantation. The sections were stained with Masson's Trichrome staining. The upper panel shows the section of PCL/HAp scaffold (right) compared to no implantation (left; Control). The lower panel shows the section of PCL (left) compared to PCL/HAp scaffold (right). Arrows showed the new bone formation. Scale bars = 2000 μm.

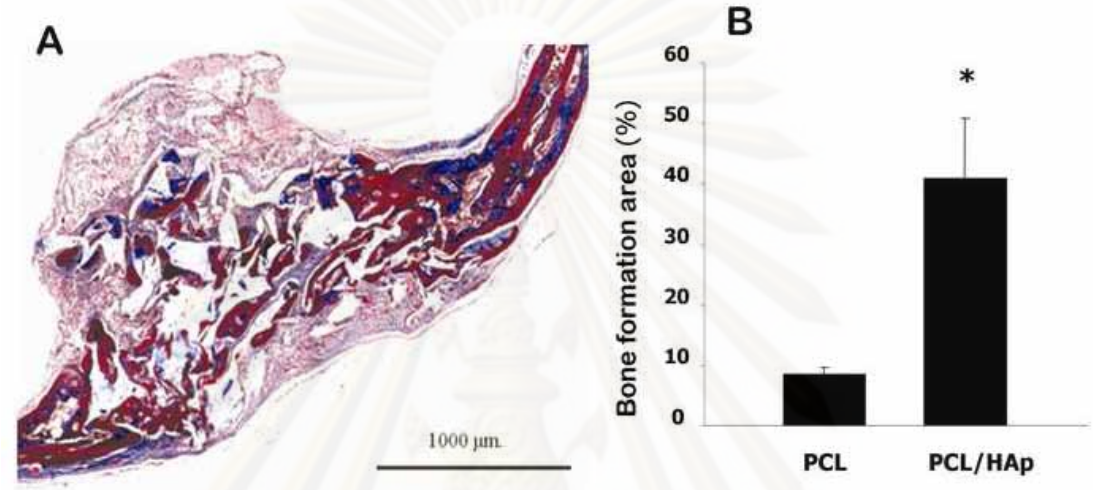


Figure 4.8. (A) Higher magnification image of implanted PCL/HAp scaffold. Scale bar = 1000 μm.

(B) Histomorphometric analysis from histological images using Scion Image analysis. The graph showed the percentage of the bone formation compared between PCL and PCL/HAp scaffolds. * Significance at $p \leq 0.05$.

Discussion

PCL has been utilized in several clinical applications, including as a temporary joint spacer and a matrix material for fabrication of a composite bone substitute (Jones et al., 2002; Elfick, 2002; Hao et al., 2003). In this study, we developed HAp-incorporated PCL scaffolds from the combined solvent casting and particulate leaching techniques for practical uses in bone tissue engineering. This combined method has been reported to be one of the suitable methods for producing porous structures suitable for bone tissue engineering (Shum et al., 2005; Wang et al., 2005). It has been shown that a scaffold with interconnected pore structure with an appropriate pore size and its distribution should facilitate the attachment and the proliferation of cells necessary for a complete tissue regeneration (Wang et al., 2005). The size of the pores in the vicinity of 150 to 500 μm has been reported to facilitate vascularization and good penetration of a new tissue (St-Pierre et al., 2005). Based on the fabrication method utilized in this work, the porosity of the scaffolds can be tailored simply by varying the amounts of the leachable particles and, at the same time, the pore size can be adjusted by using the porogens of varying dimensions. This flexibility puts the combined solvent casting and particulate leaching techniques as a suitable method for fabricating porous scaffolds for bone tissue engineering (Phillips et al., 2006; Lebourg et al., 2008).

The excellent in-growth of bone cells has been reported with a scaffold of which pore size was greater than 400 μm. (St-Pierre et al., 2005). By controlling the size of the porogen (i.e., sucrose) in the range of 400-500 μm, the size of the pores generated within the prepared PCL/Hap scaffolds certainly met this criterion. However, the addition of sucrose, after having been leached out, could also affect the density values of the resulting scaffolds. The obtained results indicated that the density of the scaffolds decreased with an increase in the sucrose content. These results are in agreement with Gong et al., 2006 who reported that the scaffolds made with sodium chloride (NaCl) particles (150-220 μm) as the porogen exhibited the porosity that increased and the density that decreased with an increase in the NaCl to polymer ratio. Based on the balance between the porosity and density of the obtained PCL scaffolds, the ones that had been prepared at the PCL to sucrose ratio of 1:10 were chosen for subsequent studies.

The addition of HAp particles within the PCL scaffolds had a strong effect on the density in an increasing manner with the HAp content, but not on the porosity of the scaffolds. Our result on the density agreed well with the report of Kothapalli et al., 2005 who showed that the density of the polylactide (PLA) scaffolds increased with an increase in the HAp content (i.e., from 0.8 to 1.3 $g \cdot cm^{-3}$ for the PLA scaffolds that contained 0-50% w/w of HAp). On the contrary to our observation, they found a monotonous decrease in the porosity of the scaffolds with an increase in the HAp content (i.e., from about 92 to 86% for the PLA scaffolds that contained 0-50% w/w of HAp). Furthermore, incorporation of HAp particles resulted in a considerable increase in the compressive rigidity of the scaffolds in an increasing manner. It is a known fact that the presence of a rigid particle within a polymeric matrix reduces the mobility of the matrix molecules (Chen et al., 2005). Kothapalli et al., 2005 also observed a monotonous increase in the compressive rigidity of the PLA/HAp scaffolds with an increase in the HAp content. Based on the balance between the density and compressive rigidity of the obtained PCL scaffolds, the ones that contained 40% w/w of HAp were chosen for subsequent studies.

The ability of a scaffold to absorb wound exudate is an important property of a functional scaffold. An amply rapid drainage of the exudate through the pore structure of the scaffold prevents the accumulation of exudate beneath the scaffold, which may finally dislodge the scaffold from the implant site due to the pressure build-up. Additionally, the passage of the exudates through the pore structure provides a possibility for certain proteins to be adsorbed on the scaffold surface. Such adsorbed proteins could play a role in facilitating the cellular in-growth. Here, the hydrophillicity of the PCL scaffolds was improved by an alkaline hydrolytic treatment. Evidently, the water retention of the scaffolds increased with increases in both the concentration of the NaOH aqueous solutions and the hydrolytic treatment time. The hydrolysis occurs at the ester linkage of PCL, giving rise to an increase in the numbers of hydrophilic carboxyl and hydroxyl groups. While an improvement in the hydrophillicity is desirable, treating the scaffolds in severe and prolonged conditions could cause the formation of cracks and more irregular pore structures to the scaffolds, which finally leads to a dramatic decrease in the mechanical integrity (Tsuji et al., 2003).

The SEM results showed that the primary human bone cells spread well on both types of the scaffolds, indicating the biocompatibility of both PCL and PCL/HAp scaffolds. However, marked increase in the cell number was observed for the cells that had been cultured on the PCL/HAp scaffolds, which implicated that the PCL/HAp scaffolds provided better support for bone cell adhesion and proliferation. The better support of the PCL/HAp scaffolds for bone cell culture should be due to the presence of HAp, a known osteoconductive material, and the marked improvement in the hydrophillicity of the scaffolds by the alkaline treatment. Similar to our results, Serrano et al., 2006 showed that the treatment of melt-pressed PCL films with 2 N NaOH aqueous solution for 2 h not only increased the hydrophilicity of the film surface, but also improved the adhesion and proliferation of endothelial and smooth muscle cells. In addition, the presence of HAp particles within the scaffolds may also facilitate specific adsorption of serum proteins that could help regulate the adhesion and proliferation of the cells.

Culture of the primary human bone cells in the 3D environment of the scaffolds could enhance the osteoblastic phenotype as evidenced by the increased expression of type I collagen and osteocalcin mRNAs of the cells cultured on the scaffolds when compared with that of the cells on TCP. However, the significant increase in the expression levels of the genes for the cells cultured on the PCL/HAp scaffolds over those for the cells on the PCL scaffolds was only observed on day 10. Because the PCL/HAp scaffolds showed a much better support for the adhesion and proliferation of the bone cells, it is possible that, during the first 10 day culturing period, the cells on such scaffolds were in the proliferation period and entered into the matrix formation phase (Wutticharoenmongkol et al., 2006). As a result, the PCL/HAp scaffolds provide a suitable environment for the proliferation and differentiation of the cells (Masi et al., 1992; Ishaug et al., 1997; Kinoshita et al., 1999). When the culture was maintained up to 21 d, significantly greater amount of calcium deposition was observed in the PCL/HAp scaffolds compared to the PCL counterparts and TCP, as determined by the alizarin red-S staining and the quantification of calcium deposition. The results positively showed that the PCL/HAp scaffolds supported the adhesion, proliferation, matrix synthesis and the differentiation of these cells. The superiority in the biocompatibility of the PCL/HAp scaffolds may result from the gradual dissolution of the incorporated HAp particles into calcium and phosphate ions that can help

regulate the behavior of the nearby cells. The PCL/HAp scaffolds then serve as a source of inorganic phosphate to enhance bone cell mineralization and the amount of the incorporated HAp particles should influence the apatitic deposition on the surface of the cells (Ciapetti et al., 2003, 2006).

Finally, the histological analysis of the scaffolds that had been implanted in the mouse calvarial defects for 6 weeks revealed that the PCL/HAp scaffolds showed better support for new bone tissue formation. The good osteoconductivity of the PCL/HAp scaffolds, when implanted *in vivo*, should be due to the presence of the HAp particles (Kim et al., 2008), since HAp and tricalcium phosphate (TCP) have been used in the treatment of bone defects such as alveolar bone augmentation (Fujita et al., 2003; Velinh et al., 2004). Despite the good osteoconductivity of HAp, implants made from pure HAp pose some difficulties in the control over their shape and size. These limitations could be alleviated with the use of the PCL/HAp scaffolds, which are much easier to handle and manipulate (Uda et al., 2006).

In conclusion, the morphological, physical, physico-chemical, mechanical and biological characteristics as well as the results from the *in vivo* implantation evaluation with the mouse calvarial defect model demonstrated that the PCL/HAp scaffolds that were obtained from the combined solvent casting and particulate leaching techniques could be an ideal scaffolding material for bone tissue engineering.

CHAPTER V
DISCUSSION AND CONCLUSION

Bone is an organic-inorganic hybrid composite of collagen and hydroxyapatite. To mimic the natural bone structure, biodegradable polymer, bioceramics and other inorganic materials can be combined. Composite materials often show an excellent balance between strength and biological activity and usually improved characteristics compared to their one component. Biodegradable materials offer several advantages over nondegradable materials. First these materials do not necessary remove by surgeons, and their degradation profile can be modulated to fit intended application. Another significant advantage is their abilities to offer researchers the opportunity to control their surface properties or construct the format of material that directly effect cellular adhesion and cellular response.

Surface characteristics of materials dictate a different adsorption rate of proteins and fluids, which in turn affect cell performance. Cell adhesion is an important parameter by which proposed tissue engineered surfaces may be evaluated to determine suitability for using materials. A cell behavior and interaction with a biomaterial surface is dependent on properties such as topography, charge, chemistry and surface energy. These properties play an important role in the cell ability to attach, adhere and spread on polymer surface and this has been referred to as the first phase of cell/material interaction. The objective of this study was to gain appropriate scaffold materials, both in surface topography and surface chemistry that can support and promote cellular adhesion and differentiation. We believed that in order to sustain the current growth and discovery in tissue engineering, a thorough understanding of the nature of interactions between biological tissues and biomaterials must occur (El-Amin et al., 2003).

We developed two kinds of three dimensional (3-D) scaffolds by incorporated biodegradable polymer with nano-hydroxyapatite to improve strength and biological properties. The first composite scaffold was PLLA/HAp constructed by electrospinning and the second was PCL/HAp prepared by solvent casting and particulate leaching technique. The synthetic nano-hydroxyapatite particle exhibited improving mechanical and biological properties of both PLLA and PCL scaffolds.

The degree of success in tissue engineering approaches is highly dependent on the properties of the scaffold used as a cell carrier. Basic scaffold design requirements include degradability, biocompatibility, high surface area/volume ratio, osteoconductivity, and suitable mechanical properties. An ideal scaffold should degrade to nontoxic products over a controlled time scale matching new tissue formation. The degradation time appropriated especially for bone repair can be engineered slowly for transfer load to the healing bone. When PLLA were degraded and then lead to build up of acidic by-products. An acidic environment were catalyzed the further degradation and cause further reduction in pH. Moreover this polymer exhibit high tensile strength and could apply in load-bearing area (Middleton and Tipton, 2000).

PCL has been widely used in tissue engineering applications because it undergoes slow hydrolytic degradation into natural metabolites. A crucial point for a scaffold to be successful, especially in bone tissue engineering, is the combination of structural/mechanical properties of polymer structure and biological activities, all of them playing a critical role in cell seeding, proliferation, and new tissue formation. Concerning degradation of the PCL/HAp composites, degradation of PCL is caused by random hydrolytic chain scission of the ester linkage. Its rate of degradation is lower than other degradable polymers, including PLLA and PLLA copolymers, because of chemical and structural characteristics (Causa et al., 2006). In vivo degradation of PCL showed a two stages pattern. The first stage involves a decrease in molecular weight without mass loss and deformation. The second degradation stage begins when the molecular weight dropped to 5,000. At that point the material broke into pieces and mass loss occurs. They therefore predicted that the material would then gradually be absorbed and excreted by the body. Afterwards the PCL ultimately excreted from the body through urine and feces. These materials did not accumulate in the any body organs (Sun et al., 2006). However two type of biodegradable polymer had different period of degradation. PCL exhibit slowly degradation time and molecular weight loss more than PLLA (Hutmacher, 2000). In orthopedic application the scaffold should degrade and maintain physical properties in load-bearing area at the same time. PCL may be proper biodegradable polymer for creating the scaffold and support this function.

From our results of PLLA/HAp showed that nano-particle HAp and polymer composites have many superior properties such as mechanical strength over PLLA scaffold. The incorporation of HAp into PLLA polymer improve biocompatibility and hard tissue integration in a way that ceramic particles, which are embedded into the polymer matrix, allowed for increased initial spread of serum proteins compared to the hydrophobic polymer surface. Wei et al., 2004 showed that the addition of hydroxyapatite particles into the composite scaffolds greatly increased protein adsorption. In addition, the basic resorption products of HAp would buffer the acidic resorption by-products of the aliphatic polyester and may thereby help to avoid the formation of an unfavorable environment for the cells due to a decrease pH (Hutmacher, 2000).

Another material was PCL/HAp scaffold that using a strategy to enhance mechanical properties by reinforce PCL with rigid HAp particles, which would also improve osteoconductivity of the polymer. In addition to this, suitable porosity achievement becomes crucial for the success of these materials as scaffolds for orthopedic application. And because the dispersion of nano-HAp particles effected to the specific surface area of HAp were more and higher hydrophilic properties than pure polymer (Heo et al., 2009). Therefore this study about HAp incorporated PCL scaffolds with primary bone cells has demonstrated that the presence of HAp on PCL substrates enhanced the osteoblast function and growth.

The present scaffold constructed by electrospinning technique that had a majority advantage of the fibers in range of nanoscale resemble features of the natural extracellular matrix (Thomas et al., 2006). El-Amin et al., 2003 suggested that the levels of ECM proteins on the surfaces may be functionally translated into an increased number of adherent cells. Because cell adhesion is strongly influencing cell behavior, a different adhesion rate on the different surfaces at the time of cell seeding. Such enhanced contact improved further steps of mineral deposition and bone formation. The cast films are not suitable for cell scaffolding because they are not porous and cannot facilitate the transport of nutrients and oxygen to the cells. Although the PLLA/HAp electrospining technique had porous structure and showed highly proliferation and differentiation more than only PLLA but this procedure had limitation. It could not control shape of scaffold and size of porous. We could not

make a scaffold with much thickness. Several techniques have been used to fabricate porous polymer scaffolds having 3-D pore structure. However from electrospinning limitation we used another technique, SCPL, can control porous size from porogen size and create shape and height of the scaffold by constructer.

The particulate-leaching method can control the pore size of scaffold by porogen have been chosen to evaluate the pore size effect. About this point the pore size and porosity of scaffolds play an important role for cell binding, migration and ingrowth and tissue regeneration. The pore size 380-405 μm. was observed with the cells and tissue. This pore size showed that the chondrocyte and osteoblast have effective cells growth and tissue regeneration (Oh et al., 2007). The creation of scaffold can access to vascular system for nutrition, gas exchange, and elimination of by-product. The diffusion of nutrients into the 3-D scaffold appropriated with interconnected macropore-structure of 300-500 μm. that enhanced the diffusion rates to and from the center of a scaffold. The optimal pore sizes for porous only hydroxyapatite have been studied before, and the estimates for optimal pore size vary from 150-500 μm (Chang et al., 2000). Apart from the porosity of the implant, the structure of the framework also affects osteoconductivity. It has been shown that porous implants must have interconnecting fenestrations to provide the space for vascular tissue required for continued mineralized bone ingrowth. The ability of a scaffold to enable the adequate delivery of nutrients to resident cells is crucial for the success of any scaffold-based tissue engineering.

A scaffold should ideally possess a porosity of 90% to allow for adequate diffusion during tissue culture and to provide sufficient area for cell-polymer interactions. Overall, pore size dimensions allow not only the fluid passage through the membrane structure, but also the trafficking of metabolites. From our PCL/HAp constructed by SCPL technique suggested that pore size approximately 400-500 μm. and porosity over 80% could enhance bone growth and vasculization in vivo.

We used primary human osteoblastic cells in this study and isolated from human bone chips, and they adapted the typical morphology exhibited by osteoblast like cells, while maintaining the expression of phenotypic marker ensured that PCL/HAp scaffolds can used in environment nearly in vivo. Our results clearly demonstrated that the interaction between primary human bone cells with PCL/HAp scaffold exhibit expression markers of differentiation and after implantation in mouse

calvarial defect showed that osteoblasts migrated into the scaffold for bone repair. We reported PLLA/HAp scaffold can support mouse osteoblast cells but only in vitro. For in vivo analysis we found that cells could attach around and grow only surface area of the scaffold (data not shown). This reason persuaded us for concentrated to study involve PCL/HAp scaffold. In conclusion we suggested that combining HAp with PCL produced by SCPL method should provided better results in a promising combination of strength biodegradability and bioactivity more than PLLA/HAp created by electrospinning technique and may be a proper scaffold to use in the future.

Future study

The PCL/HAp scaffold demonstrated that it could support bone repair in vivo especially in mouse. The next step we will try to implant this scaffold in another animal model. And for developing the potential of this scaffold, we will use the concept of tissue engineering that contained cells, proper scaffold and biological factor. For solving the inadequate cell source, we will develop by culturing this scaffold with adult human mesenchymal stem cells from dental pulp or adipose tissue. When we will provide the appropriate inductive agent and find the best source of the cells. We can repair the bone loss by these scaffolds and may help improving the quality of life in our patients in the future.

The generation of designer 3-D porous biodegradable constructs is attractive for the development of skeletal repair strategies, not only as a much needed scaffold/filler for bone regeneration but also as cell delivery structures. The development of supercritical fluid mixing technology to generate polymer scaffolds of defined porosity and degradation characteristics has provided new platform technologies that can be applied to a variety of cells including osteoprogenitors.

Furthermore the biodegradable scaffolds can be enhanced with adhesion motifs and growth factors and extending the range as well as specificity of biodegradable scaffolds (Howard et al., 2002). Bone substitutes have been successfully developed through the interaction of osteoconductive growth factors and/or osteogenic cells into an osteoconductive scaffolding matrix. However important barrier toward the clinical application of tissue-engineering bone grafts is inadequate biological attractive

substance and availability of a sustained mineralizing cell source. In order to solve these problems in the future, we will develop these scaffolds by adding soluble growth factors such as bone morphogenetic protein (BMP) especially BMP-2 and BMP-7 or using gene delivery of osteogenic transcription factors, such as RUNX2/Cbfa1. In particular gene expression may help mineral deposition. From Philips et al., 2006, they demonstrated that the osteogenic potential of Runx2 expressing for fibroblasts is highly dependent on scaffold properties. Therefore the developed PCL/HAp scaffold with appropriated biological growth factor may be made this scaffold as an osteoinductive material and used to speed the healing process after a fracture.

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