ประสิทธิภาพของโครงร่างพอลิคาโปรแลคโตน/ไฮดรอกซีแอปาไทต์เคลือบด้วยพอลิไดเอลิวไดเมททิวแอมโมเนียมคลอไรด์/พอลิโซเดียมสไตรีนซัลโฟเนต/พอลิสไตรีนซัลโฟนิกแอซิดโคมาลลิอิกแอซิด : การศึกษาในห้องปฏิบัติการและในสัตว์ทดลอง

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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The Efficacy of $(PDADMAC/PSS)_9$/PSS-co-MA coated Polycaprolactone /Hydroxyapatite scaffold improve new bone formation : An in vitro and in vivo study

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การปลูกถ่ายอวัยวะเป็นสิ่งที่จำเป็นสำหรับการฟื้นฟูบูรณะข้อบกพร่องที่มีขนาดใหญ่ การปลูกถ่ายโดยการใช้กระดูกของผู้ป่วยเองนับเป็นรักษาที่เหมาะสมในทางอุดมคติที่สุด แต่วิธีนี้ยังคงมีข้อจำกัดอยู่บางส่วน ดังนั้น คณะผู้วิจัยจึงมีความพยายามที่จะพัฒนาวัสดุโครงร่างพอลิคาโปรแลคโตน/ไฮดรอกซีแอปาไทต์ให้เป็นทางเลือกในการนำไปใช้ทดแทนการปลูกถ่ายกระดูกจากตัวผู้ป่วยเอง โครงร่างพอลิคาโปรแลคโตน/ไฮดรอกซีแอปาไทต์นี้ได้รับการศึกษาซึ่งได้พิสูจน์แล้วว่ามีลักษณะและคุณสมบัติเชิงกลซึ่งสามารถสนับสนุนการสร้างกระดูกได้ ทางคณะผู้วิจัยจึงใช้หลักการของการเคลือบฟิล์มบางทีละชั้นด้วยโพลิไดเอลิวไดเมททิวแอมโมเนียมคลอไรด์ (พีดีเอดีเอ็มเอซี) โพลิโซเดียม-4-สไตรีนซัลโฟเนต (พีเอสเอส) และโพลิ-4-สไตรีนซัลโฟนิกแอซิดโคมาลเลอิคโซเดียวมซอลล์(พีเอสเอสโคเอ็มเอ) บนผิวของโครงร่างโพลิเมอร์ เทคนิคการเคลือบผิวทีละชั้นนี้ได้รับการพิสูจน์โดยการเคลือบบนพื้นผิวแก้วและผิวเรียบของพีซีแอลโพลิเมอร์แล้วว่า มีคุณสมบัติความชอบน้ำและส่งเสริมการแปรสภาพของเซลล์สร้างกระดูก อีกทั้งยังสามารถเหนี่ยวนำให้เกิดการสร้างกระดูกในความวิการของกระดูกกะโหลกศีรษะหนูทดลองใน 6 สัปดาห์

ทางคณะผู้วิจัยได้ทำการศึกษาโดยนำคุณสมบัติการเคลือบผิวทีละชั้นด้วยพอลิอิเล็กโทรไลต์นี้ เคลือบไปบนผิวของโครงร่างพอลิคาโปรแลคโตน/ไฮดรอกซีแอปาไทต์นี้ และศึกษาความสามารถในการยึดเกาะของเซลล์ การเพิ่มจำนวนของเซลล์ การแปรสภาพของเซลล์สร้างกระดูกของหนู (เอ็มซีสามทีสาม-อีหนึ่ง) จากผลการศึกษาพบว่าการเคลือบพีเอสเอสโคเอ็มเอ สามารถเพิ่มค่าเอ็มทีที และการตกตะกอนของแคลเซียมได้ และจากการศึกษาการสร้างกระดูกในสัตว์ทดลองโดยฝังโครงร่างพอลคาโปรแลคโตน/ไฮดรอกซีแอปาไทต์ที่เคลือบด้วยพีเอสเอสโคเอ็มเอในกระดูกขาของสัตว์ทดลอง พบว่ามีผลทางจุลกายวิภาคศาสตร์เนื้อเยื่อของการสร้างกระดูกที่มากขึ้น จากการศึกษาทั้งทางห้องปฏิบัติการและในสัตว์ทดลอง การเคลือบผิวของโครงร่างพอลิคาโปรแลคโตน/ไฮดรอกซีแอปาไทต์ด้วยพอลิอิเล็กโทรไลต์ พีเอสเอสโคเอ็มเอ สามารถเป็นหนึ่งในทางเลือกของวัสดุที่จะนำไปใช้ในงานวิศวกรรมเนื้อเยื่อกระดูกได้

ภาควิชา.....ทันตกรรมประดิษฐ์.....ลายมือชื่อนิสิต.....

สาขาวิชา.....ทันตกรรมประดิษฐ์.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEYWORDS : POLYCAPROLACTONE HYDROXYAPATITE SCAFFOLD/ PEM/ BIOMATERIAL/ POLYSTYLENE-CO-MALEIC ACID

PORNPEN JITTIVARANGKOOL : THE EFFICACY OF $(PDADMAC/PSS)_9$/PSS-CO-MA COATED POLYCAPROLACTONE/HYDROXYAPATITE SCAFFOLD IMPROVE NEW BONE FORMATION : AN IN VITRO AND IN VIVO STUDY. ADVISOR : PROF. PRASIT PAVASANT, D.D.S., Ph.D., CO-ADVISOR : ASSOC.PROF. PIYAWAT PHANKOSOL, D.D.S., Ph.D., 66 pp.

Grafting material is necessary for large defect reconstruction. Autograft is an ideal treatment for bone reconstruction, however some limitation still. Therefore, development in an alternative 'scaffold' to replace autograft comes to attention. Polycaprolactone/hydroxyapatite (PCL/HAp) scaffold which had been proved morphology and mechanical properties could support bone formation. This study developed the PCL/HAp scaffold by the concept of polyelectrolyte multilayer coating with Poly(4-styrenesulfonic acid-co-maleic acid) sodium salt (PSS-co-MA), Poly(diallyldimethyl-ammonium chloride) (PDADMAC) and Poly(sodium 4-styrene sulfonate) (PSS). This technique had been proved on planar material as glass and PCL membrane that had better wettability and could promoted osteoblast differentiation and also induce bone formation in murine calvarials defects at 6 weeks.

This PEM coating (layer by layer) was combined onto the PCL/HAp scaffold surface and evaluated the ability of cell adhesion, proliferation, differentiation and mineralization of MC3T2-E1 cells. The results showed PSS-co-MA coating scaffold surface expressed increasingly MTT and calcium deposition. For in vivo new bone formation, PSS-co-MA coating PCL/HAp scaffold was implanted into circular defect of rat femur bone. Histological analysis of bone formation increased from 1 weeks and completed in 6 weeks. From in vivo and in vitro testing indicated that the $(PDADMAC/PSS)_9$/PSS-co-MA coated scaffold could be the material of choice for bone tissue engineering.

Department : Prosthodontics	Student's Signature .....
Field of Study : Prosthodontics	Advisor's Signature .....
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LIST OF ABBREVIATIONS

AP	Alkaline phosphatase
BCP	Biphasic calcium phosphates
Cscf	Coated scaffolds
DBM	Demineralised bone matrix
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
FBS	Fetal bovine serum
HAp	Hydroxyappatite
HMDS	Hexamethyldisilazane
LbL	Layer-by-Layer
MSCs	Mesenchymal stem cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-dipheyl tetrazolium bromide
nHAp	nano-Hydroxyapatite
Nscf	Non coated scaffolds
PBS	Phosphate buffered saline

PCL	Polycaprolactone
PDADMAC	Poly(diallyldimethyl-ammonium chloride
PEG	Poly(ethylene glycol)
PEM	Polyelectrolyte multilayer
PGA	Poly (glycolic-acid)
PLA	Poly(lactic-acid)
PLLA	D- and L- Poly(lactic aid)
PNPP	p-nitrophenyl phosphate
PPF	Poly (propylene fumarate)
PSS	Poly(sodium 4-styrene sulfonate)
PSS-co-MA	Poly(4-styrenesulfonic acid-co-maleic acid) sodium salt
SCPL	Solvent Casting & Particulate Leaching
SEM	Scanning electron microscopy
SFF	Solid free form fabrication
TCP	Tricalcium phosphate
VEGF	Vascular endothelial growth factor

CHAPTER I
INTRODUCTION

Each year, the incidence of bone resection procedure tends to be increasing by several causes like trauma, abnormal development and especially cancer[1]. Most of these cause large defects that cannot self-reconstruct as it was. Consequently, the challenging treatment is required, such as bone reconstruction by using autograft, allograft, xenograft or other synthetic grafting material[2]. Although the ideal treatment is autograft to the recipient site, but there are a lot of limitations in this way due to limited donor site supply, risk of complication of secondary site pain and a massive suffering[3]. One way to solve these problems is developed synthetic polymer using as bone substitution.

Synthetic polymers emerge as a new option supplement of bone formation in porous 3D structure called “scaffolds” as previous study that show the success in new bone formation and expressed ostegenic markers by using polycarprolactone (PCL) collaborated with hydroxyapatite (HAp) in porous 3D scaffold[4]. This novel fabricated scaffold, by using a solvent casting and particulate leaching technique with 400-500 μm porosity[5], can support growth and differentiation of bone cells both *in vitro* and *in vivo*[4]. The biodegradation rate of the scaffold was match to the growth rate of new bone tissue that are required for bone tissue restitution[2]. Moreover, the process of new bone formation not only involved in suitable material framework, but also related to the surfaces which play an important role in a biological system for most biological reactions that occur at surfaces and interfaces[6]. So, we continue to develop the active surfaces which can provoke specific cellular responses and direct new tissue regeneration by Polyelectrolyte multilayer (PEM) technique via Layer-by-Layer (LbL) deposition of alternating adsorption of polyanions and polycations dipping on the surface of materials[7]. The electrostatic attraction between oppositely charged polyelectrolytes is the main driving force for multilayer build up. *In vitro* study of this PEM with Poly(4-styrenesulfonic acid-co-maleic acid) sodium salt (PSS-co-MA),

Poly(diallyldimethylammonium chloride) (PDADMAC) and Poly(sodium 4-styrene sulfonate) (PSS) coating can enhance osteoblast differentiation, improve calcium deposition and also possible to accelerate osseointegration process[8]. However, the ability of this PEM surface modification *in vivo* model is not revealed.

The objective in this study was to investigate the ability of PEM *in vitro and in vivo* model cooperated with PCL/HAp scaffold.

Research questions

1. Whether $(PDADMAC/PSS)_9$ / PSS-co-MA PEM coated PCL/HAp scaffold support osteoblasts functions including cell adhesion, proliferation, differentiation and calcium deposition *in vitro* better than PCL/HAp scaffold alone.

2. Whether $(PDADMAC/PSS)_9$/PSS-co-MA PEM coated PCL/HAp scaffold support the new bone formation *in vivo* model better than PCL/HAp alone.

Research objectives

To examine the osteoinductive effect of $(PDADMAC/PSS)_9$/PSS-co-MA PEM coated PCL/HAp scaffold in new bone formation *in vitro* and *in vivo* model.

Hypothesis

Ho_1: $(PDADMAC/PSS)_9$/PSS-co-MA PEM coated PCL/HAp scaffold can increase osteoblasts functions similar to PCL/HAp scaffold *in vitro*.

HA_1: $(PDADMAC/PSS)_9$/PSS-co-MA PEM coated PCL/HAp scaffold can increase osteoblasts functions better than PCL/HAp scaffold *in vitro*.

Ho_2: The amount of new bone formation in $(PDADMAC/PSS)_9$/PSS-co-MA PEM coated PCL/HAp scaffold is not different than that found in PCL/HAp scaffold alone in *in vivo*.

HA_2: The amount of new bone formation increased in $(PDADMAC/PSS)_9$/PSS-co-MA PEM coated PCL/HAp scaffold compared to that found in PCL/HAp scaffold alone in *in vivo*.

Ho_3: New bone formation in $(PDADMAC/PSS)_9$/PSS-co-MA PEM coated PCL/HAp scaffold occur in the same rate compared to that found in PCL/HAp scaffold alone in *in vivo*.

HA_3: New bone formation in $(PDADMAC/PSS)_9$/PSS-co-MA PEM coated PCL/HAp scaffold occur with faster rate compared to that found in PCL/HAp scaffold alone in *in vivo*.

EXPECTED BENEFITS

This study will provide the information about the PEM technique and the advantage of this technique to be used in craniofacial tissue engineering. We hope the development of this scaffold surfaces will bring an alternative material for bone regeneration can serve for several cases that compromise of autograft to help in delay healing, non-union or critical-sized defect scenarios in the future.

CHAPTER II

REVIEW OF RELATED LITERATURE

Biology of bone

Bone is a dynamic biological tissue which composed of organic and inorganic elements. By weight, bone is approximately 20% water. Dry bone, weight, is made up of 65–70% inorganic calcium phosphate and 30–35% an organic matrix of fibrous protein and collagen. The cellular components are osteogenic precursor cells, osteoblasts, osteoclasts and osteocytes[9].

Osteoblasts are mature bone forming cells which have differentiated from mesenchymal stem cells(MSCs)[10]. Osteoblasts are responsible for skeletal architecture in two ways: (1) deposition of bone matrix and (2) regulation of osteoclast activity[11]. They secrete osteoid, the unmineralized organic matrix that subsequently undergoes mineralization, giving its bone strength and rigidity. It is composed of 90% type I collagen and 10% ground substance, which consists of noncollagenous proteins, glycoproteins, proteoglycans, peptides, carbohydrates, and lipids. The mineralization of osteoid by inorganic mineral salts provides bone with its strength and rigidity. The inorganic content of bone consists primarily of calcium phosphate and calcium carbonate, with small quantities of magnesium, fluoride, and sodium. The mineral crystals form hydroxyapatite (HAp; $Ca_{10}(PO_4)_6(OH)_2$), which precipitates in an orderly arrangement around the collagen fibers of the osteoid. The initial calcification of osteoid typically occurs within a few days of secretion but is completed over the course of several months[9, 11]. As their bone forming activity nears completion, some osteoblasts are converted into osteocytes whereas others remain

on the periosteal or endosteal surfaces of bone as lining cells. Osteoblasts also play a role in the activation of bone resorption by osteoclasts[12].

Osteocytes are mature osteoblasts trapped within the bone matrix. Each osteocyte form a network of cytoplasmic processes extends through cylindrical canaliculi to blood vessels and other osteocytes. These cells are involved in the control of extracellular concentration of calcium and phosphorus, as well as in adaptive remodeling behavior via cell-to-cell interactions called mechanotransduction[9].

Osteoclasts are multinucleated, derived from fusion of mononuclear hemopoietic precursors. The primary function of osteoclasts is to secrete hydrolytic acids and proteolytic enzymes, which erode bone extracellular matrix (ECM) under the influence of chemical signals. This process controlled by hormonal and cellular mechanisms. These cells function in groups termed “cutting cones” that attach to bare bone surfaces, releasing hydrolytic enzymes and dissolve the inorganic and organic matrices of bone, appearance in shallow erosive pits on the bone surface called Howship lacunae[10, 12].

There are three primary types of bone: woven bone, cortical bone, and cancellous bone. Woven bone formed during embryonic stage and in some disease as hyperparathyroidism and Paget’s disease and also found in fracture healing in callus formation. This bone is composed of randomly arranged collagen bundles and irregularly shaped vascular spaces lined with osteoblasts. Woven bone is remodeled and changed to be cortical or cancellouse bone. Cortical bone also called compact or lamellar bone that vascularized invaded from periosteal and endosteal surfaces. Cortical bone formed like a flat bone plate internal and external surfaces of bone. The unit of cortical bone called osteon as haversian system which compose of cylindrical shape of lamellar bone that surrounds longitudinally oriented vascular channels called haversian canals. Osteon also connected

horizontally to adjacent osteons by Volkmann canal that communicated to other osteon within compact bone. Each of osteons are tight together refer to the mechanical strength of compact bone. Cancellous bone, some called trabecullar bone or spongy bone consists of a network of bone trabecale and hematopoietic elements like spongy or honeycombed. This bone lies between the internal and external plate of compact bone. trabeculae orientated perpendiculated to external forces to provide structural support. Cancellous bone plays an important role in remodeling of endosteal bone[9].

Bone healing

In this way, these bone cells play a critical role in both bone formation and bone repairing. The process occurs via an intramembranous (direct) or endochondral (indirect) process depends on anatomic site. Intramembranous ossification occurs during embryonic development of the cranial vault bones when The primitive mesenchymal cells are transformed into osteoprogenitor cells and directly ossification into mature osteoblasts leading to the formation of the bone with all of its histological characteristics[13]. This process occurs typically in the calvarial bones, mandible and the clavicle, whereas the epiphysial growth plate in the appendicular skeleton is characterized by the intracartilagenous bone formation. In this process the primitive mesenchymal cells differentiate in a two-step process into mature bone. First, the mesenchymal cells transform into chondroblastst, form collagen and other elements of bone matrix, become ossified, and lead to mature bone[14]. In bone regeneration process requires more such as a morphogenetic signal, responsive host cells, a suitable carrier to specfic sites, etc. that serve as a scaffolding for the growth host cells and well vascularization[15].

The process of bone healing that occurs in fractured long bones is influenced by a variety of systemic and local factors. Healing occurs in three distinct but overlapping stages: 1) the early inflammatory stage; 2) the repair stage; and 3) the late remodeling stage. In the inflammatory stage, a hematoma develops within the fracture site during the first few hours and days. Inflammatory cells as macrophages, monocytes, lymphocytes, and polymorphonuclear cells and fibroblasts infiltrate the bone by prostaglandin mediation. This results in the formation of granulation tissue, ingrowth of vascular tissue, and migration of mesenchymal cells. The primary nutrient and oxygen supply of this early process is provided by the exposed cancellous bone and muscle. The use of antiinflammatory or cytotoxic medication during this 1st week may alter the inflammatory response and inhibit bone healing. During the repair stage, fibroblasts begin to lay down a stroma that helps support vascular ingrowth. As vascular ingrowth progresses, a collagen matrix is laid down while osteoid is secreted and subsequently mineralized, which leads to the formation of a soft callus around the repair site. In terms of resistance to movement, this callus is very weak in the first 4 to 6 weeks of the healing process and requires adequate protection in the form of bracing or internal fixation. If improper immobilization, ossification of the callus may not occurs, and an unstable fibrous union may develops instead. Fracture healing is completed during the remodeling stage in which the healing bone is restored to its original shape, structure, and mechanical strength. Remodeling of the bone occurs slowly over months to years. Adequate strength is typically achieved in 3 to 6 months. Bone grafts are also strongly influenced by local mechanical forces during the remodeling stage. The density, geometry, thickness, and trabecular orientation of bone can change depending on the mechanical demands of the graft[9]. In 1892, Wolff first popularized the concept of structural adaptation of bone, noting that bone placed under compressive or tensile stress

is remodeled. Bone is formed where stresses require its presence and resorbed where stresses do not require it. This serves to optimize the structural strength of the graft. Conversely, if the graft is significantly shielded from mechanical stresses, as in the case of rigid spinal implants, excessive bone resorption can potentially occur and result in a weakening of the graft. This potential disadvantage of instrumentation needs to be balanced with the beneficial effects.

Bone grafts

Bone graft is the second most common transplantation tissue. Annually, more than 500,000 bone grafting procedures in the United States and 2.2 million worldwide are happening in order to repair bone defects in several fields including dentistry[16]. The gold standard of bone-grafting is harvesting autologous cortical and cancellous bone from the iliac crest. All other forms of bone grafting have disadvantages compared to autograft and such as insufficient and traumatic of donor site their use is sub-optimal. Technological evolution along with better understanding of bone-healing biology, however, Several methods of reconstructing bone defects are available using allograft, demineralized bone matrix, hydroxyapatite calcium phosphate, other related growth factors and surface modification like PEM, coating, sol-gel etc.[2].

Bone regeneration with bone grafting occurs by the three essential elements of osteogenesis, osteoinduction and osteoconduction that bonding between host bone and grafting material called osteointegration.

Osteogenesis is the ability of the graft to produce new bone, this graft materials contain viable cells with the potential to differentiate into bone- forming cells as inducible osteogenic precursor cells or the ability to form bone as osteoprogenitor cells. This process

related the presence of live bone cells in graft which participate in the early stages of the healing process to unite the graft with the host bone. Osteogenesis is a property found only in fresh autogenous bone and in bone marrow cells, although some studies, authors of radiolabeling studies of graft cells have shown that very few of these transplanted cells survive[9].

Osteoconduction serve as scaffold or template for bone healing process which allows for neovascularize ingrowth and filtration of osteogenic precursor cells into the graft site. Osteoconduction is the physical properties of graft that are found in cancellous autografts and allografts (demineralized bone matrix, hydroxyapatite, collagen and calcium phosphate).

Osteoinduction is the ability of graft material to induce stem cells to differentiate into mature bone cells. This process is generally associated with the presence of bone growth factors within the graft material or as a supplement to the bone graft. Bone morphogenic proteins and demineralized bone matrix are the principal osteoinductive materials. Autograft and allograft bone also have some osteoinductive properties.

Autograft

Autologous bone provides optimal osteoconductive and osteogenic properties. The most common donor site is iliac crest as it provides a good quality and quantity cancellous autograft. Havesting autograft bone from iliac typically is easy to access, however surgical procedure usually complicated by residual pain and cosmetic disadvantages. Other limitations include elderly or paediatric patients and patients with malignant disease. In addition autograft harvesting is associated with a 8.5—20% of complications including haematoma formation, blood loss, nerve injury, hernia formation, infection, arterial injury, ureteral injury, fracture, pelvic instability, cosmetic defects, tumour transplantation, and

sometimes chronic pain at the donor site. Furthermore, it may fail in clinical practice as osteogenic cellular elements do not survive transplantation.

Allograft

Allograft is the most frequently chosen bone substitute as secondary choice from autografts. For the past decade, using rate of allograft increase about one-third of bone grafts performed in the United States. Some disadvantages are variable clinical results of grafting and carry the risk of transferring viral diseases. The processing of allograft tissue lowers this risk but, that can significantly weaken the biologic and mechanical properties initially present in the bone tissue. Allograft can be prepared from fresh, frozen or freeze dried forms, cortical or cancellous. Fresh allografts are rarely used as they might ignite an immune response or transmit diseases.

Bone graft substitutes

A bone-graft substitute should be: osteoconductive, osteoinductive, biocompatible, bio-resorbable, structurally similar to bone, easy to use, and cost effective. A large number of bone-graft alternatives are currently commercially available for use. They vary in composition, mechanism of action, and special characteristics. Synthetic bone scaffold should maintain its mechanical properties for at least 1–3 months after implantation and then should be totally resorbed through metabolic pathways after 12–18 months so that it does not impeded tissue ingrowth and regeneration.

Demineralised bone matrix

Demineralised bone matrix (DBM) produces from decalcification of cortical bone, remains the trabecular collagenous structure of original bone. This material serves as a biological osteoconductive scaffold and osteoinductive despite loss of some structure and strength that contribute from mineralize bone. DBM can reduce the potential of

transinfection and immunogenic rejection, and some growth factors still remain. There are several forms of DBM usages as a gel, moldable putty, paste, bone chip or injection type. Therefore, DBM is widely use and clinical results are not surely uniform. Thus, DBM can be used as a bone graft extender rather than bone graft substitute[10, 17-19].

Synthetic bone substitutes

Synthetic bone substitute is an alternative choice that approach to represent the autograft and allograft. This synthetic bone graft posses not all ideal bone graft material. Ideally synthetic bone graft substitutes should be biocompatible, show minimal fibrotic reaction, undergo remodeling and support new bone formation. Synthetic bone graft should have biomimetic bone characteristic and similar strength to cortical and cancellous bone being replaced. This also includes modulus of elasticity, toughness maintainance and fatique fracture. The disadvantages in clinical usage are unpredictable resorption and results and some inflammatory response like foreign body. Considerable modified of composite material such ceramic or hydroxyapatite to overcome the several complications[2].

Ceramics

Calcium phosphate ceramics were introduced more than 40 years ago as bone substitutes. Ceramics scaffolds are made from calcium phosphate that has been used in dentistry and in orthopaedics since the 1980s[20, 21]. Synthetic ceramic alone is not osteogenic or osteoinductive properties, but can be induce biologic response of bone. These materials are considered bioactive as they bond to bone and enhance bone tissue formation. The bioactivity of these ceramics has been attributed to the similarity of their composition and structure compared with the mineral phase of bone. The most common types of calcium phosphate materials investigated for synthetic bone scaffold development

are: hydroxyapatite (HAp);$Ca_{10}(PO_4)_6(OH)_2$, tricalcium phosphate (TCP); $Ca_3(PO_4)_2$, biphasic calcium phosphates (BCP), and multiphasic bio-glasses. On the basis of the composition and stoichiometry of a calcium phosphate ceramic, important physical properties such as degradation rate, modulus, and process ability can be changed. TCP material has degradation rate is too rapid, while synthetic HAp degrade too slowly to allow native tissue integration. These properties motivated the development of BCP and bio-glasses which have change degradation rates; more commonly β-TCP and HAp in a composite ceramic.

Biologic/synthetic composite grafts

Composite synthetic grafts approach to be an alternative choice to fullfil the disadvantage of single materials. The use of composite graft that contains osteogenic cells and osteoinductive properties for bone grafting is more controlled and effective combinations without the disadvantages found with autograft. The choice of the appropriate bone substitute scaffold should be based on several parameters having in mind that the gold standard remains the autograft. The incorporation of such factors in an osteoconductive scaffold it's a very promising option. The tissue-engineering is the one choice will create biological scaffolds, and also development the product ex vivo tissue engineered substitutes for use.

Bone tissue engineering

Tissue engineering was originally defined from a broad and general perspective as "the application of the principles and methods of engineering and life sciences towards the fundamental understanding of structure function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve functions[22]. The goal of tissue engineering is to surpass the limitations of

conventional treatments based on organ transplantation and biomaterial implantation[23].Tissue engineering offers a new option to supplement existing treatment regimens for several parts of the body and the oral cavity offers distinct advantages to the tissue engineer, such as ease of observation and accessibility[24]. Today, tissue engineering is driven by multidisciplinary research like several groups of study in many fields in tissue engineering, as its show in Figure 1. There are specific problem studies related to tissue engineering e.g. design and fabrication of scaffolds, cell isolation and characterization, cell proliferation, cell differentiation, bioreactors, surface modification, etc. Whether it can be modified biomaterials, biochemistry, immunology, polymer processing or others both in the industry and academic to solve the problem that still facing in patient treatment.

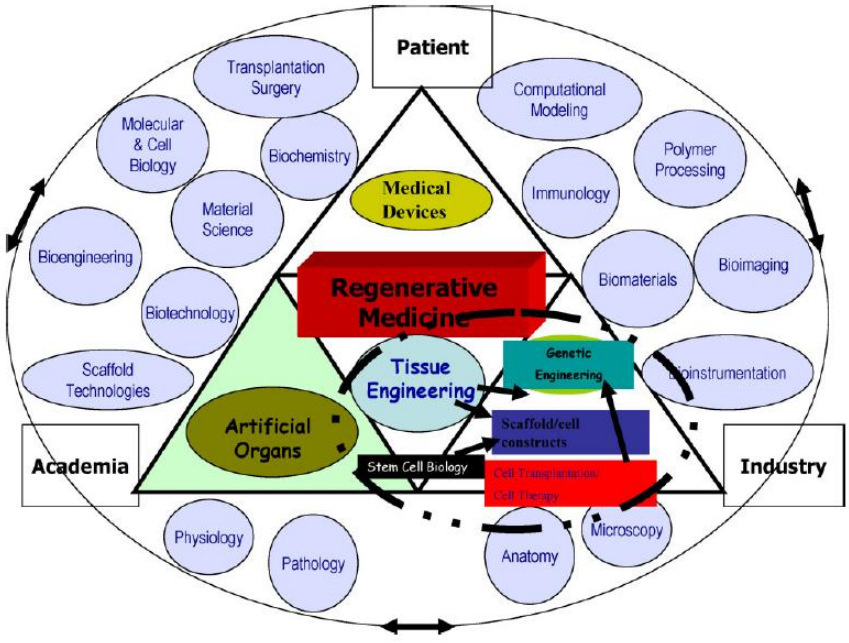


Figure1 Overview of multidisciplinary field of tissue engineering and regenerative medicine[22].

The development of biomaterials for tissue engineering applications has recently focused on the design of biomimetic materials that are capable of eliciting specific cellular responses and directing new tissue formation mediated by biomolecular recognition, which can be manipulated by altering design parameters of the material. Biomolecular recognition of materials by cells has been achieved by surface and bulk modification of biomaterials via chemical or physical methods with bioactive molecules or incorporated with surface modification.

Common Polymer in bone tissue engineering

For bone biomimetics, the primary objective in bulk material selection criteria for bone tissue engineering is to mimic native bone tissue. The bulk material composition plays a critical role in the overall success of scaffolds. The bulk material must be biocompatible, biodegradable and have appropriate mechanical properties for load bearing applications. A variety of materials have been investigated for synthetic bone scaffolds including metals, ceramics, polymers, and composites of these[25].

Polymers and polymer- ceramic composites are the principle materials investigated for the development of synthetic bone scaffolds. Common polymers which have been investigated for bone repair applications include polyesters, poly (propylene fumarate)(PPF), poly(ethylene glycol) (PEG), poly(orthoesters), polyanhydrides, and polyurethanes.

Polyester

Polyesters are the most commonly researched polymers for bone regeneration applications. Aliphatic polyesters such as poly(lactic-acid)(PLA), poly (glycolic-acid)(PGA), poly(caprolactone) (PCL), and their copolymers are the most commonly utilized polymers in

bone tissue engineering[3, 26-28]. These polymers have been FDA-approved and utilized in a wide variety of clinical applications such as sutures, systemic drug delivery, spinal fusion cages, coronary stents, fixation screws, and nerve conduits[29]. This is likely because there are several FDA-approved polyesters with extensive clinical history. Another favorable characteristic of polyesters is the easily copolymerized with other materials to change the degradation rate[30].

poly(lactic-acid)

PLA is the cyclic dimer of lactic acid, which exists as two isomers: D- and L-Poly(lactic aid) (PLLA) is 37% crystalline with a melting temperature of 60–65°C, and a degradation time of up to several years. However, due to the low modulus of PLA, it must be either copolymerized with a higher modulus polymer, or made into a composite with a different material.

poly (glycolic-acid)

PGA, the simplest linear aliphatic polyester is highly crystalline (45–55%), has a high melting point (220°C), and a glass transition temperature of 35°C. PGA alone has a high modulus (7 GPa), and completely degrades in vivo within 4–6 months[30]. Like PLA, PGA has also been used in a bone tissue engineering applications. Both PGA and PLA scaffolds has been investigated as a slow delivery carrier for growth factors in several in vitro and in vivo studies, and demonstrated the ability to promote healing and osseointegration compared with control scaffolds[31]. However, most researchers copolymerize PLA and PGA to increase control over degradation rates and mechanisms for a specific application. A description of common copolymers utilized in drug delivery for bone tissue engineering applications is provided below.

Polycaprolactone

Poly(ε-caprolactone) (PCL) is another semi-crystalline polyester with a glass transition and melting temperature of approximately -60°C and 60°C, respectively[10]. The degradation time for PCL is similar to PLA. Because of it relatively slow degradation rate, and high modulus compared with other FDA approved biodegradable polyesters, it is well suited for orthopedic and drug delivery applications. PCL is formed through ring-opening polymerization of the cyclic monomer ε-caprolactone. Catalysts such as stannous octate are used to catalyse the polymerization as show in Figure 2. PCL has FDA approval for bone and cartilage repair[32]. The interesting property of PCL is the kinetics of biodegradation which is considerably slower than other aliphatic polyesters[33]. PCL is a bioresorbable polymer with potential applications for bone and cartilage repair. PCL is more stable in ambient conditions. It is significantly less expensive and is readily available in large quantities.

Additionally, PCL degradation products are easily resorbed through metabolic pathways and do not produce local acidic environments as opposed to polylactides and glycolides. The local acidic environment produced by polylactides and glycolides may effects the stability of a protein or other bioactive molecule in the preparation and delivery stage[34]. Biodegradation of PCL is susceptible to hydrolysis (Figure 3)[35]. The hydrolytic degradation mechanism is complex and involves several phenomena. The degradation occurs through two phase; the first stage, a decrease in molecular weight without mass loss and deformation and afterwards the materials gradually lost strength and broke into pieces. In the second stage, low molecular weight PCL pieces metabolized by unknown process which mainly involves intracellular phagocytosis[36, 37] and ultimately excreted from the body through urine and feces. Due to all of above and its low cost, sustained

biodegradability, availability at low molecular weight, more stable in ambient condition, and readily available in large quantities[33], PCL is very attractive.

Much research is currently focused on the use of PCL biocomposites and co-polymers of PCL with both natural and synthetic polymers [21-31]. PCL scaffolds have previously been created with a variety of solid free form fabrication (SFF) techniques that can produce proper porosity and mechanical properties and can support bone generation in pig condyle[26]. For bone scaffolds this can provide good implant bonding and maintaining biological and mechanical integrity[38, 39]. One group has recently demonstrated enhanced osteoblast functionality in vitro and bone formation in vivo as a result of controlled delivery of calcium phosphates and growth factors from PCL scaffolds[40-43]. In another novel approach, PCL scaffolds were functionalized with laminin-derived peptide sequences known to promote cell adhesion and proliferation (i.e., RGD, YIGSR) and in vitro analysis with adipose-derived stem cells indicated that peptide grafting to PCL materials enhanced cellular adhesion and proliferation[44].

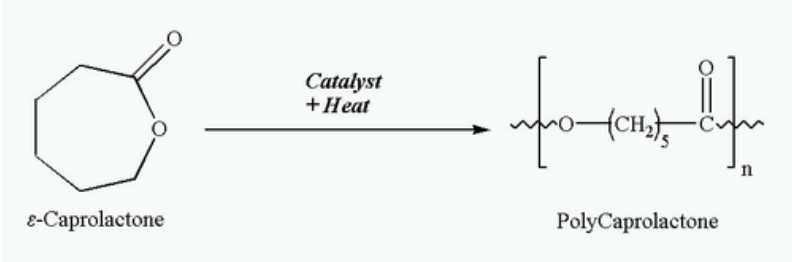


Figure 2 Ring opening polymerization of **ε**-caprolactone to polycaprolactone

(Ref: http://www.Synthetic Biodegradable as Medical Devices (MPB archive, Mar 98).html)

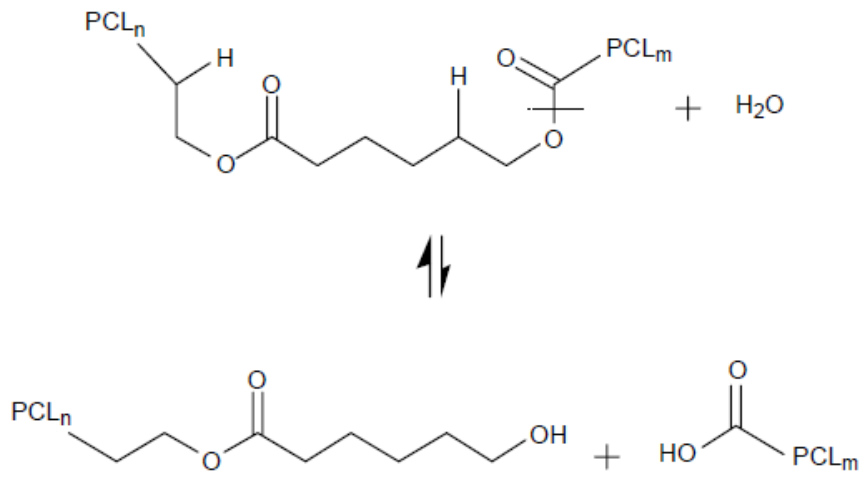


Figure 3 Typical polyester hydrolysis reaction[35].

Mechanisms of polymer biodegradation

Biodegradation of polymers can be generally categorized as bulk or surface degradation. In bulk degradation, the rate of water penetration exceeds the degradation and solubilization rate of surface molecules resulting in a bulk material degradation and consequently the loss of macroscopic mechanical properties of the scaffold. In surface degradation, the surface molecules degrade and solubilize faster than the water penetration rate resulting in surface erosion while bulk material maintains its structural integrity. Polyesters, polyether–esters, and polyester–amides are classified as bulk-degraders and typically yield first order release profiles, while polyanhydrides and polyorthoesters are classified as surface eroders which typically yield zero-order profiles. Most polymers utilized for synthetic bone scaffolds are semi-crystalline polyesters, in which case there are two primary mechanisms of degradation. First, the polymer is degraded through random chain scission characterized by a reduction in molecular weight. Random chain scission means that any ester bond in the chain has equal probability of cleavage via hydrolysis. This is in contrast to chain-end scission reaction which, as the name implies, occurs at the terminus

of the polymer chain. No weight loss is observed in this initial phase which covers a molecular weight of 200 kDa to 5 kDa. Second, when the molecular weight is reduced to a sufficiently low number (5 kDa has been suggested), polymer fragments diffuse out of the bulk polymer matrix characterized by a measurable weight loss. A decrease in chain scission is likely in the second phase due to an increase in polymer crystallinity since chain scission preferentially occurs on amorphous regions of the polymer. In practice, however, biodegradation of a polymer scaffold cannot be characterized this simply as the degradation rate and mechanisms will also depend on (1) the micro and nanoscale structure of the scaffold, (2) the polymerization of multiple polymers/polymer types, and (3) the presence of hydrolytic accelerators or suppressors. The *in vivo* degradation rate of polyesters is significantly enhanced compared with *in vitro* degradation rates. This is assigned in part to the optimum concentration of ester degrading enzymes such as lipases in the human body. To mimic the *in vivo* environment, many researchers utilize bacterial enzymes such as Pseudomonas cepacia lipase at higher than physiologic concentrations. Selection of a polymer for synthetic bone scaffold development pivots on some key design criteria such as: (1) degradation rate is comparable to tissue development/ ingrowth rate, (2) biodegradation products are biocompatible, and (3) polymer processability (i.e., ease of copolymerization, covalent attachment of key molecules).

Polycaprolactone/hydroxyapatite scaffold

In recent years, biodegradable Polycaprolactone/hydroxyapatite scaffolds have been interested used for bone tissue engineering[4, 26, 35, 45]. The ideal porous scaffolds should be biocompatible, biodegradable and absorbable. In addition, suitable microstructure (including porosity, pore size and interconnection between pores), sufficient

mechanical strength retaining for a period and good cell-scaffold interaction are also the necessary requirements for a three-dimensional scaffold applied in bone tissue engineering[46, 47]. Considering that the extracellular matrix of natural bone consists of a complex organic, inorganic composite, significant attention has been paid to polymer/ceramic composite applied in bone regeneration[48].

For Hydroxyapatite, calcium–phosphate minerals, is the primary constituent (60%) of bone[10]. To mimic the physical attributes of bone, synthetic scaffolds must have a high degree of porosity. When ceramics such as tricalcium phosphate (TCP;$Ca_3(PO_4)_2$, hydroxyapatite (Hap; $Ca_{10}(PO_4)_6(OH)_2$ and biphasic calcium phosphates (BCP) are formed into porous scaffolds, the macroscopic mechanical properties are inadequate for load bearing surfaces due to the inherent brittleness of the ceramics. This seriously limits their clinical relevance as synthetic bone scaffolds[33, 49, 50]. Calcium phosphate ceramics are an obvious choice and have consistently demonstrated excellent cellular and tissue responses *in vitro* and *in vivo*. Calcium–phosphate ceramics were introduced more than 40 years ago as bone substitutes. These materials are considered bioactive as they bond to bone and enhance bone tissue formation. The bioactivity of these ceramics has been attributed to the similarity of their composition and structure compared with the mineral phase of bone. Research on TCP materials has revealed that the degradation rate is too rapid *in vivo*, while synthetic versions of HAp degrade too slowly to allow native tissue integration[10]. Synthetic HAp has been shown to be biocompatible and promotes osteoblast adhesion and migration/infiltration *in vitro*. Dozens of calcium phosphate formulations have been developed and investigated for their bioactivity.

Gui et al., has recently summarized a model for the biointeraction between CaP materials and bone demonstrated that CaP materials (specifically biphasic CaP) have

superior stability and *in vivo* osteogenic properties compared with autologous bone grafts in critical-sized bone defects. Short- and long-term *in vitro* and *in vivo* studies have confirmed that CaP materials induce osteogenic differentiation (osteoinduction), promote MSC migration (osteoconduction), and allow for bony tissue ingrowth and integration (osseointegration)[49, 51]. Several groups have shown favorable results for this therapy in bone regeneration. A literature review indicates that a pore size in the range of 10– 400 μm may provide enough nutrient and osteoblast cellular infusion, while maintaining structural integrity[52, 53]. Additionally, there have been several successful fabrication techniques (i.e., soaking in simulated body fluid, combustion synthesis, compression/sintering)[10]. Therefore, there are increasing interests in the preparation and investigation of porous PCL/nHAp composite as scaffolds for bone tissue engineering. The addition of hydroxyapatite (HAp) particles to PCL, which would improve the mechanical properties, especially increasing its stiffness[54] and promote osteoconductivity[55], offers a way to provide a material more generally suitable as a scaffold for hard tissue replacement. [4] PCL/HAp composite materials often show an excellent balance between strength and toughness and usually show improved characteristics when compared to their separate components. Many studies that interested in PCL/HAp scaffold are summarized in table 1.

Table 1. PCL/HAp composites scaffold for different development.

materials	Development technique	Main finding	Reference
PCL/HAp	Precision extrusion deposition (PED) process technique	*In vitro*; good mechanical properties and biocompatible	[56]
PCL/nHAp	Melt-molding/porogen leaching technique	70% porosity decrease compressive modulus Properties can retain to 6 months and slightly change depended on amount of nHAp	[57]
PCL/HAp	(1)conventional blending (2)grafting of PCL on HAp particles	Mechanical properties in wet state was lower than dry state both method and more in blending method	[45]
PCL/HAp	electrospinning	Greater proliferation rate of MC3T3-E1 cells	[58]
PCL/HAp	Phase inversion and casting	20-32% HAp in PCL improvement in mechanical performance of scaffold	[59]

Scaffold morphology and design

To determines the suitability of a particular material, the considerations for scaffold design are hence complex and include material composition, architecture, structural mechanics, surface properties, degradation properties and by products, together with the composition of any added biological components which must be regarded[22]. Several requirements have been identified as: (1) the scaffold should possess interconnecting

pores of appropriate scale to favour tissue integration and vascularisation, (2) be made from material with controlled biodegradability so that tissue will eventually replace the scaffold, (3) have appropriate surface chemistry to favour cellular attachment, differentiation and proliferation, (4) possess adequate mechanical properties to match the intended site of implantation and handling, (5) should not induce any adverse response and, (6) be easily fabricated into a variety of shapes and sizes[23, 60]. Bearing these requirements in mind, the following lists should be considered.

1. Material selection

Depending on the specific intended application of the matrix, whether for structural support, drug delivery capability, or any osteoinduce agent for well suited to the final structure. Choices of matrix material include polymers, ceramics, and composites of the two. Polymers can impart a wide variety of physicochemical and mechanical properties to a formed matrix, the synthesis of which can include natural polymers, such as type I collagen, hyarulonic acid, chitosan, etc. Ceramics is a material made from an inorganic that can possess acrystalline structure which typically has a high resistance to deformation but brittle in nature. Combination of polymers and ceramics into one composite material could capture the advantages of each component like degradation time and physical properties[28]. Selection of a polymer for synthetic bone scaffold development pivots on some key design criteria such as: (1) degradation rate is comparable to tissue development/ingrowth rate, (2) biodegradation products are biocompatible, and (3) polymer processability (i.e., ease of copolymerization, covalent attachment of key molecules). As above introduction , several of the most commonly utilized polymers and copolymers for synthetic bone scaffolds is provided[10]. Polylactic acid (PLLA), their copolymers (e.g. PLGA) and polycaprolactone (PCL) are the most commonly used polymers for tissue engineering scaffold applications.

The degradation products of these polymers are removed by natural metabolic pathways[23, 60].

2. Biocompatibility and Biodegradability

Biocompatibility is the ability of the scaffold to perform in a specific application without eliciting a harmful immune or inflammatory reaction[61]. There are normally three-stages tissue responses when a polymeric scaffold was implanted in a tissue. Stage 1, the first 1 to 2 weeks after implantation, is characterized by acute and chronic inflammatory responses. Acute inflammation happened from the first minute and an hour to days, depending on the extent of the injury. Chronic inflammation results from long-term presence of inflammatory stimuli and is confined to the implantation site. In general, the stage 1 response is independent of the degradation rate of the polymer. When the numbers of monocytes and macrophages increase, stage 2 start. The fibrous encapsulation of the foreign material initiated, the length of this stage depended on the rate of biodegradation of the scaffold. Fibrous encapsulation continues in stage 3. And time taking depended on the function of the degradation rate of the polymer. Slowly degrading polymers in a stage 3 response happens for weeks to months, whereas polymers degraded rapidly as short as 1 to 2 weeks[62].

In bone tissue engineering, the immune response is a major concern because degradation products cause failure in many orthopedic implants. Therefore, a stage of a biomaterial design must consider is the initial properties of the scaffold with its degradation products that can effect on the host. Materials intended for implantation should be minimized the intensity and duration of response with a controllable degradation rate as well as the rate of natural regeneration. The breakdown products should be non toxic and easily excreted from the body via metabolic pathways or renal filtration system[63]. The

mechanism of degradation of aliphatic polyester microspheres is a hydrolytic mechanism, which strongly supported by the detailed efforts of Vert et al. and *in vitro* and *in vivo* studied by Visscher et al. and Ikada et al.[62]. Mechanisms can be generally categorized as bulk or surface degradation, as show in Figure 4.

In bulk degradation, the rate of water penetration exceeds the degradation and solubilization rate of surface molecules resulting in a bulk material degradation and consequently the loss of macroscopic mechanical properties of the scaffold. In surface degradation, the surface molecules degrade and solubilize faster than the water penetration rate resulting in surface erosion while bulk material maintains its structural integrity. Polyesters, polyether–esters and polyester–amides are classified as bulk-degraders, while polyanhydrides and polyorthoesters are classified as surface eroders as described previously. The *in vivo* degradation rate of polyesters is significantly enhanced compared with *in vitro* degradation rates. This results was assigned by esterdegrading enzymes such as lipases in the human body[10].

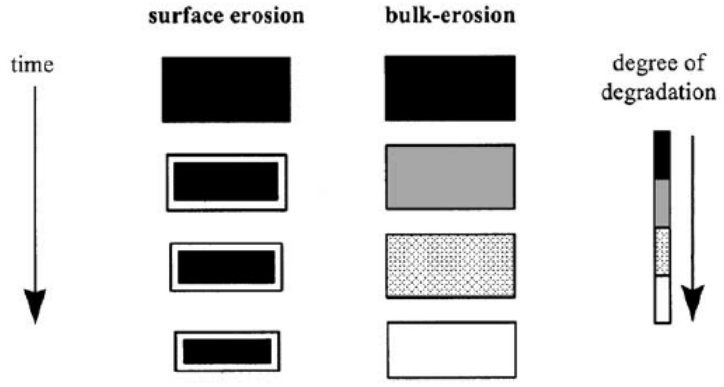


Figure 4 Schematic illustration of the changes a polymer matrix undergoes during surface erosion and bulk erosion [64].

3. Mechanical properties

The mechanical properties of the scaffold should be designed to meet the specific requirements of the tissue to be regenerated at the defect site. Furthermore, at the time of implantation, the scaffold should have sufficient mechanical integrity to allow for handling by the clinician, to be able to withstand the mechanical forces imposed on it during the implantation procedure and survive under physical conditions. Some research has shown that a synthetic bone scaffold should maintain its mechanical properties for at least 1–3 months after implantation and then should be totally resorbed through metabolic pathways after 12–18 months so that it does not impeded tissue ingrowth and regeneration[10]. For proper tissue regeneration without significant deformation, a scaffold should provide a mechanical modulus of 10 to 1500 MPa for hard tissue and 0.4 to 350 MPa for soft tissue[65].

4. Scaffold architecture

It is well recognized that the pore size of scaffolds plays an important role for cell binding, migration and ingrowth, and tissue ingrowth and regeneration. Generally, it was reported that the large pore size or porosity of the scaffold can allow effective nutrient supply, gas diffusion and metabolic waste removal but lead to low cell attachment and intracellular signaling. Many researchers have reported optimum pore size ranges for the different kinds of cells or tissues, for example, the pore sizes of ~5 μm for neovascularization , 5–15 μm for fibroblast ingrowth , ~20 μm for hepatocyte ingrowth , 20–125 μm for skin regeneration , 70–120 μm for chondrocyte ingrowth , 40–150 μm for fibroblast binding , 45–150 μm for liver tissue regeneration , 60–150 μm for vascular smooth muscle cell binding , 100–300 μm for bladder smooth muscle cell adhesion and ingrowth , 100–400 μm for bone regeneration , and 200–350 μm for osteoconduction[66], depending

on the suitable site of usage. The study by Chuenjitkuntaworn et al.[4] showed that when porosity is increased mechanical properties will decrease, so the proper porosity which can withstand the maximum mechanical properties is range of 400–500 μm.

Manufacturing technology

To gain the several factors influence the success in tissue engineering as review above. There are several different techniques have been introduced to fabricate the porous conventional biodegradable polymer matrices. The most common techniques are list as followed.

- Solvent Casting & Particulate Leaching (SCPL)
- Gas foaming
- The supercritical fluid-gassing process
- Fibre meshes/fibre bonding
- Phase separation
- Melt moulding
- Solution Casting
- Emulsification/Freeze-drying
- CAD/CAM Technologies

Several papers reviewed each technique[10, 23, 46]. In this study use Solvent Casting & Particulate Leaching (SCPL). This technique allows the preparation of porous structures with regular porosity by producing the solution of polymer into a suitable organic solvent like polylactic acid could be dissolved into dichloromethane, polycaprolactone in chloroform, and combination with adding the porogen. The porogen can be an inorganic salt like sodium chloride, crystals of saccharose, gelatin spheres or paraffin spheres. The

solvent is allowed to fully evaporate leaving behind a polymer matrix with salt particles embedded throughout in the mold and immersed in a bath of a liquid suitable for dissolving the porogen, water in case of sodium chloride, saccharose and gelatin or an aliphatic solvent like hexane in case of paraffin. Once the porogen has been fully dissolved a porous structure is obtained[23]. 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[67]. The advantages are simple fabrication and do not require any complicated equipment. In contrast the disadvantages are time comsuming and the limitation to of thin structures, irregularly shaped pores, and insuficient interconnectivity[46].

Polyelectrolyte multilayer films (PEMs)

Surface modification is alternative technique to develop in tissue engineering material for specific such as lubricity, protein resistance, enhanced protein adsorption, degradation protection, inhibit of cell adhesion, enhancement of cell attachment and growth, and antibacterial properties[68]. Additionally, the surface energy may play a role in attracting particular proteins to the surface of the material and, in turn, this will affect the affinity of the cells to the material[15].

Definition and formation

Polyelectrolyte multilayer films(PEMs) is the one technique to fabricated active surface. This technique was first introduced by Decher et al. over a decade ago[7]. It was used for microencapsulating pancreatic islet with sodium alginate and poly(L-lysine) to prolong survival of islets and circumvent immune rejection problem[68]. By definition

“polyelectrolytes are polymers that contain relatively high degree of ionizable groups along their backbone chain. Polyelectrolytes can be cationic, anionic or amphophilic (contains both cationic and anionic groups that are present in the same or different monomer units)”. Polyelectrolytes can be synthesized by polymerization of monomer units or by modification of the polymer to induce charges on the monomer repeat units. Polyelectrolytes which can be found in nature include proteins, polysaccharides and nucleic acids. The presence of ionic groups on the monomer repeat unit has a tremendous effect on the properties of polyelectrolytes. They generally exhibit higher water solubility, expanded hydrated dimensions and a higher sensitivity to ionic strength and pH than nonionic polymers. Polyelectrolytes have been found uses in many biomedical applications such as dental restoratives, where the polyelectrolytes are ionically cross linked with the aid of multivalent cations (Zn^{2+}, Mg^{2+}, Al^{3+}, Ca^{2+}) leading to insoluble cement matrix[68].

Build up polyelectrolyte multilayer films

These films are prepared by exposing substrates to solutions of synthetic or natural polyelectrolytes based on an electrostatic layer-by-layer (LbL) self- assembly process[7]. When a charged surface is exposed to opposite charge polyelectrolyte, the polymer sticks to the surface physically due to electrostatic attraction. The binding is strong due to the several interaction points with the surface. After being exposed to the oppositely charged polymer, the surface is then immersed in a rinse solution to wash off the loosely bound polymer. A solution of polymer with the opposite charge to the first polymer is then deposited to form another layer. The substrate may be immersed and rinsed, in an alternating fashion, in the two polyelectrolyte solutions to make ultrathin films (1-100 nm in thickness), as shown in the Figure 5.

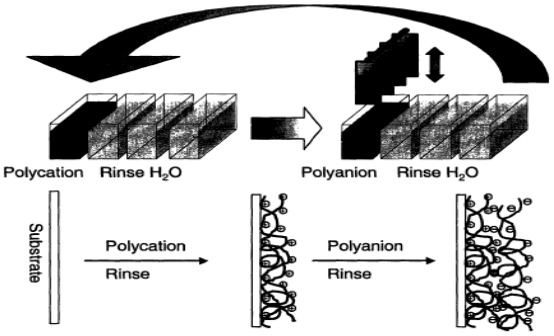


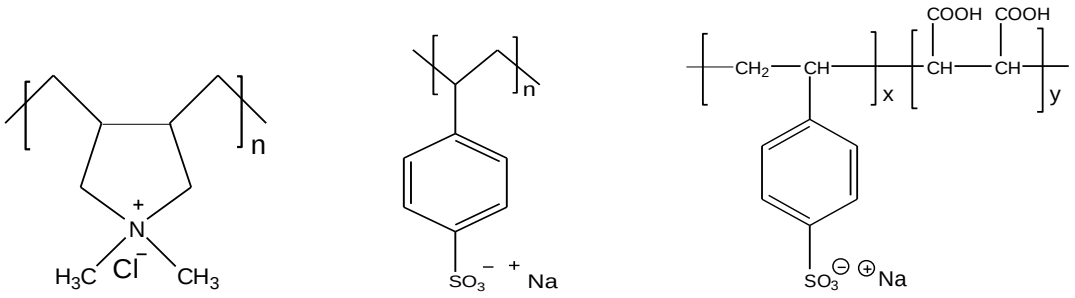
Figure 5 Assembly process for layer-by-layer polyelectrolyte films form by alternately dipping a substrate in a poly cation and polyanion solution[69].

There is an active interest in using multilayer polymer thin films for tissue engineering. In tissue engineering, cell adhesion to implant surface is critical because cell adhesion occurs before other biological events including cell spreading, cell migration and differentiation and cell function. Cell adhesion is closely related to the surface properties of biomaterials. It is commonly accepted that the adhesion of cells to solid substrata is influenced by several substratum surface properties, such as wettability, surface charge, roughness and topography. Many surface modification techniques have been used to produce various surface properties of polymers. Most conventional materials do not meet the criteria for serving as tissue engineering scaffolds. Surface modification is an effective approach to alter biological interactions of a particular material to develop appropriate scaffolds. Since surface modification only changes the outermost surface composition of a biomaterial, its bulk properties do not change. In addition, surface modification can provide accessible and chemical functional groups for the immobilization of drugs, enzymes, antibodies or other biologically active species for a variety of biomedical applications. The

factors determining the scaffold quality and its interaction with cells and/or tissues involve the quality of the biomaterial, the processing of scaffold, post-treatment and the presence of contamination. Interactions of the scaffold with cells are determined by its structure, the presence of pores, pore size, geometry, distribution, surface texture (roughness, pattern, orientation) and surface chemical properties (such as hydrophilicity, free energy, ionic interaction, electric charge). Cell attachment and proliferation strongly depend on the chemical and physical properties of the surface of a tissue engineering scaffold. Many researchers prepared various specific surfaces in order to study the correlation. The use of polyelectrolyte multilayers in the biomedical field is attracting the attention of surface scientists due to the flexibility and ease with which PEM can be used to modify surfaces and change interfacial properties. Physical adsorption of polymer may lead to higher density of an anchoring polymer as compared to chemical surface reactions or grafting, Charged surfaces using poly(diallyldimethylammonium) and poly(styrene sulfonate) were investigated for selective protein arrays. Coated particles modified with (PSS) and 4th generation poly(amidoamine) dendrimer showed decreased human serum protein adsorption. Others have studied the influence of the polyanion type, pH and salt on protein interaction with multilayers[68]. Polymers are typically flexible molecules, the resulting superlattice architectures are somewhat fuzzy structures, but the absence of crystallinity in these films is expected to be beneficial for many potential applications.

Poly (diallyldimethylammonium chloride) (PDADMAC) which is strong cationic polyelectrolyte consist of positive charges along the backbone chain. In contrast, Poly (sodium 4-styrene sulfonate) (PSS) is strong anionic polyelectrolyte and consist of negative charges along the backbone chain. Both PDADMAC and PSS were used in many studies to investigate factors influencing for the properties and structure of polyelectrolyte multilayer

films[70-73]. This is because their strong polyelectrolytes have ionic charges that are largely independent of the solution pH condition[74]. As for PDADMAC polycations, its hydrophobic ring structure is stiff and consequently difficult to rotate, both in water and in air. As a result, the outer layer containing the quaternary ammonium end groups stays hydrophilicity. Hydrophilic property of the polycations will influence the next surface of the subsequent adsorbed PSS layer[75]. Poly(4-styrenesulfonic acid-co-maleic acid) sodium salt (PSS-co-MA) is a copolymer of PSS and maleic acid (MA). Building a multilayer film with copolymer consisting of both weak and strong polyelectrolyte pendant groups may obviate the need for chemical cross-linking to improve the stability of weak polyelectrolyte multilayers. In such a case, the strongly charged groups can form electrostatic linkage which can enhancing film stability, while the weakly charged groups can be used to alter multilayer properties because they are responsive to external pH changes[76]. The ionization of the weak polyelectrolyte is dependent on the pH. At high pH, PSS-co-MA was used as anionic polyelectrolyte which included strong anionic group (sulfonate group) and weak anionic group (carboxylic group). Although PSS-co-MA has been recently used as cation-exchange, it was not employed in this technique for supporting osteoblast functions. A plenty of researches try to modify surfaces for developing biocompatible polyesters wheather it be cell seeding or growth factors[29, 77], interestingly by polyelectrolyte multilayer also investigated. Natural polyelectrolytes such as poly(L- lysine) and hyaluronic acid (HA), studied extensively, render films bioinert and biodegradable. The chemical structures of the polyelectrolyte were showed in Figure 6.



PDADMAC PSS PSS-co-MA

Figure 6 Chemicals structure of polyelectrolytes used in this research[8].

PEM coating for tissue engineering substrate

For application in bone biomaterial fields and tissue engineering, the PEM film are widely used to modify the surface of several material as Luxsana Limsavarn and coworker showed their study in PDADMAC/PSS-co-MA on medical devices[78]. The layer-by-layer deposition of polyelectrolytes multilayers was used for the formation of hydrophilic coatings inside microfluidics channels. Ladam et al. have shown by their studies of polyelectrolyte multilayers on silica that the structure of the first few deposited layers ~~is~~ influenced by the substrate and that it takes a few layers before a “linear deposition regime” is adopted[79, 80]. There are many studies that try to develope the PEM technique coating by several type of polymer as summarized in table 2. As recently, Angwarawong et al. proved the ability of PDADMAC/PSS/PSS-co-MA on glass slide and Ti surface to support osteoblast function both *in vitro* and *in vivo*. This coating enhanced osteoblast proliferation and promoted mineralization that improved osteointegration[8].

Table2. PEM coating on materials for tissue engineering appoication

PEM films	Material substrate	Main finding	Reference
PSS-co-MA/PAH	Silicon wafer and quart and glass	Film thickness decrease with increase pH	[76]
PSS/PDADMAC	Poly(ethylene terephthalate)	Coating density follow coating layer number. PSS outermost layer more stable to shear force.	[81]
PSS-PAA/PAH	Polyethelenimine	Increase PSS ratio resulting in decreasing film thickness, surface roughness, Increase hydrophobicity and IgG adsorption	[82]
PDADMAC/Clay platelets	PA hydrogel ICC scaffolds	Improve hydrogel to support cell adhesion of bone marrow support cell	[83]
PGA-PLL	Oral Prosthesis	Coating can increase wettability substrate and was not degraded by saliva for awhile	[84]

This objective of this study was to fabricate PEM film of PDADMAC/PSS)9/PSS-co-MA on 3D PCL/HAp scaffold. The ability of osteoinductive effects were investigated *in vitro* and *in vivo*.

CHAPTER III

RESEARCH METHODOLOGY

Preparation of Polycaprolactone/Hydroxyapatite scaffolds

Preparation of hydroxyapatite (HAp) followed the technique of Shih et al[85]. Briefly, HAp was prepared by co-precipitation method. In the typical procedure, 2 g of $CaHPO_4.2H_2O$ (as a phosphorous source) and 0.79 g of $CaCO_3$ (as a calcium source) were dissolved in 1 M HNO_3 25 ml under gentle stirring at 70 ˚C for 2 h and the solution was kept to pH 2. 200 ml of 1 M tris-base solution was then poured into the mixture generating the precipitation at pH 7. The precursor solution was stirred vigorously to yield a homogeneous product. The product was then filtered off and washed several times with deionized water. After centrifugation, the resulting material was freeze-dried for 48 h to obtain the fine powder products.

PCL/HAp scaffolds were fabricated by solvent casting and particulate leaching techniques followed by Chuenjitkuntaworn et al. by dissolving polycaprolactone (PCL; Aldrich; Mw 580,000 g/mol) in Chloroform [Labscan (Asia), Thailand] which used as the solvent at ratio of 0.286g : 1ml. Hydroxyapatite powder was added to the PCL-chloroform solution to obtain a 40 wt% of HAp in PCL and mixed well by stirring at room temperature for 10 min. Sucrose (Fluka Chemika, Switzerland) was used as the porogen, 400–500 μm particles, sucrose was added to the PCL/HAp suspensions at the ratio was 1:10 w/w. The mixture was poured into a glass plate mould and allowed to dry for 24 h. After drying, the scaffolds were immersed in distilled water for 2 days to dissolve away the porogen with the changes of distilled water every 4 h and removed from glass mold. Then, the scaffolds were leaving at room temperature scaffolds to allow the evaporation of residual water for another 2 days. Finally, scaffolds were treated with 1M sodium hydroxide (NaOH; Ajax Finechem, Australia) aqueous solution at 37°C for 6 h to create the hydrophilic surface. The alkaline treated scaffolds were washed thoroughly in distilled water and dried in vacuo for 48 h[4].

After that, Preparation PCL/HAp scaffold into coil shape size 12 mm diameter, high 1mm for using in laboratory experiment and size 3 mm diameter, high 1mm for using in animal experiment.

Fabication of Polyelectrolyte Multilayer films of $(PDADMAC/PSS)_9$/PSS-co-MA on PCL/HAp Scaffolds

Polyelectrolyte multilayer films were constructed by sequentially deposited layers of PDADMAC (Aldrich; Mw 200,000-350,000 g/mol) followed by PSS (Aldrich; Mw 70,000 g/mol) as the positive charge and negative charge, respectively to create 9 layers of PDADMAC-PSS. Subsequently, the 10^{th} layer was conformed with the negatively charge polyelectrolyte, PSS-co-MA (Aldrich; Mw 20,000 g/mol). Briefly, the prepared PCL/HAp scaffold were alternatively immersed in 10mM PDADMAC containing 0.1M NaCl for 5min, rinsed with deionized distilled water three times for 2 min each, followed by immersing in 10mM PSS containing 0.1M NaCl for 5 min with intermediate triple rinses using distilled water until the ninth layer was formed. For the tenth layer, the scaffolds were immersed in 10mM of PSS-co-MA containing 0.1M NaCl at pH10 for 30 min, then rinsed three times with distilled water (pH 10) and left them dried at room temperature. The fabrication method diagram was shown in Figure 7.

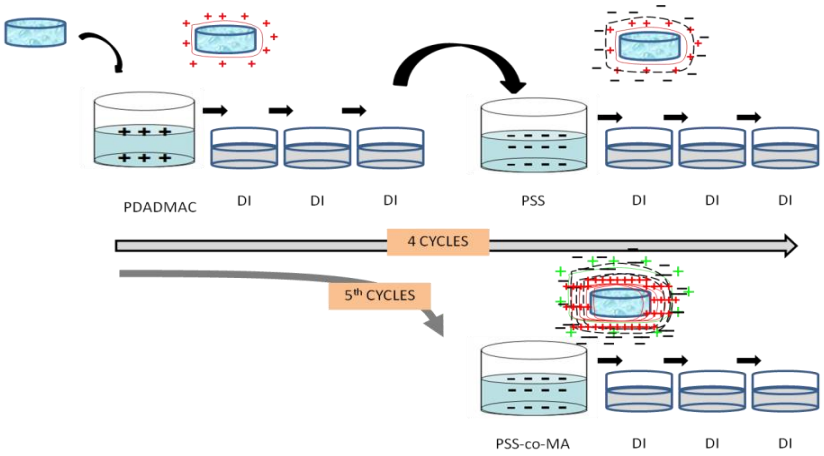


Figure 7 Fabrication of Polyelectrolyte Multilayer films (PEMs)

Finally, the preparation scaffolds divided into 2 groups of coated and non-coated scaffolds were sterilization by dipping in 70% v/v ethanol for 30 min and washed with sterilized de-ionized water and incubated in Dulbecco's modified Eagle's medium (DMEM; GIBCO) containing 15% fetal bovine serum (FBS; ICP Biologicals, New Zealand), 2 mM L-glutamine, 100 units/mL penicillin, 100 lg/mL streptomycin, and 5 lg/mL amphotericin B (GIBCO) in 37°C incubator overnight before the day of experimental test.

Cell Culture

MC3T3-E1 cells (ATCC CRL-2593), the immortalized cell line derived from mouse calvarium tissue, were used in the experiments. Cells were maintain in minimum essential medium (HyQ® MEM/EBSS, Hycone, Logan, Ultah, USA) supplemented with 10% fetal bovine serum (FBS, ICP biologicals, Henderson, Auckland, New Zeland), 2 mM L-glutamine, 100 unit/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Gibco,Grand Island, New York, USA) and grew under standard culture condition (at 37°C in 100% humidity and 5% CO_2). The medium was changed every other day. The cells between 18 to 22 passages were used. All experiments were performed triplicate.

Determination of cell attachment using scanning electron microscopy (SEM)

MC3T3-E1 cells were seeded on coated and non-coated scaffolds at a density of 50,000 cells/well. Cover glass slide was used as a control. After cultured for 1, 4 and 16 h, the unattached cells were removed, rinsed with phosphate buffered saline (PBS) and fixed with 3% glutaraldehyde solution (Fluka, Steinheim, Germany) for 30 min. After fixation, cells were rinsed with 0.1M PBS twice, dehydrated in an alcohol series (30%, 50%, 70%, 90% & 100% ethanol) for 2 min at each concentration followed by critical point dried using 100% hexamethyldisilazane (HMDS, Fluka, Steinheim, Germany) for 5 min. The thin layer of gold was sputter-coated on the surface of all the samples before examination the morphology of cell attachment under scanning electron microscope (JSM 5410LV, JEOL, Japan).

Cell proliferation (MTT assay)

MC3T3-E1 cells were seeded at the density of 50,000 cells/ well on coated and non-coated scaffolds and cultured in culture medium (10%MEM) as described. At culture for 1, 3 and 5 days, the cell number was measured using MTT assay to determine the rate of cell proliferation. MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-dipheyl tetrazolium bromide) solution was prepared at 5 mg/ml by dissolved MTT solution (USB Corporation, Cleveland, OH, USA) in 10% serum culture medium without phenol red. To perform the assay, the cultures were washed with PBS, followed by addition of MTT solution into each well and incubated for 30 min at 37°C. After incubation, the formazan product was dissolved by 1 ml mixing of glycine buffer (pH = 10) (125 µl/well) and dimethyl sulfoxide (DMSO, Sigma-Aldrich, Seelze,Germany) (900 µl/well). The optical density of this colored solution in each well was measured using Thermospectronic Genesis10 UV-visible spectrophotometer at a wavelength of 570 nm.

Alkaline phosphatase activity (ALP activity)

MC3T3-E1 cells were seeded into the scaffold and cultured for 5, 10 and 15 days. The cultures were rinsed with PBS and lysed with alkaline lysis buffer (10 mMTris-HCl, 2 mM $MgCl_2$, 0.1% Triton-X100, pH 10). The lysis solution from each culture was divided into 2 parts. The first half of volume was mixed with p-nitrophenyl phosphate (PNPP substrate; Zymed, Invitrogen, Carlsbad ,CA ,USA) and 0.1M aminopropanol in 2mM $MgCl_2$ was added into the substrates. After incubated at 37 ºC for 15 min, the above mixture was added with 0.1 M NaOH to stop the reaction and the absorbance at 410 nm was measured using UV-vis spectrophotometer. The other half of volume prepared to protein assay using a bicinchoninic acid (BCA) protein assay (BCA™, thermo Scientific, Rockford, IL, USA), The mixed solution was incubated at 37 ºC for 2 min and then measured the amount of total protein at the absorbance 562 nm.

Alizarin red-S staining and calcium quantification

Calcium deposition was quantified by Alizarin Red staining (Alizarin Red S – certified, Sigma, St.Louis, MO, USA).To examine the bone nodule formation, the MC3T3-E1 cells were seeded at a density of 50,000 cells/well on the scaffold specimens. After 3 days in culture, the medium contained an osteogenic supplement, ascorbic acid (50 lg/mL; Sigma) and β-glycerophosphate (5 mM; Sigma) was changed. Well plate was incubated further for another 21 days with changing the medium on every other day. Each specimen was rinsed with PBS after removal of the culture medium, fixed with cold methanol for 10 min and washed with deionized water and immersed in 2 ml of 1% Alizarin Red solution dissolved in 1:100 (v/v) ammonium hydroxide/water mixture (pH = 4.2) for 3 min. The amount of calcium was quantified by destaining with 10% cetylpyridinium chloride monohydrate (Sigma, St. Louis, MO, USA) in 10 mM sodium phosphate at room temperature for 15 min and spectrophotometically read at 570 nm.

Animal experimental test

This experiment was designed to observe the bone formation in rat femur bone at 1, 2, 4 and 6 weeks after scaffold implantation. The experiment was carried out on a 200-300 g male Wistar Rats (National Laboratory Animal Centre, Mahidol University, Thailand) under general anesthesia with Avertin by intraperitoneal injection. The operation start with local infiltrated and intramuscular infiltrated with anesthesia. Incision and flap opening, a circular defect on femur bone (3 mm in diameter) was created by trephine bur. The twelve for each of PEM coated scaffold and non-coated scaffolds were implanted into defect randomly in twenty four rats, as showed in Figure 8. The wound was closed with a 3-0 resorbable vicryl suture (Ethicon Inc., a subsidiary of Johnson and Johnson). Housing and feeding of the animals according to standard animal care protocols, was approved by the Animal Care and Use Ethical Committee, Faculty of Medicine, Chulalongkorn University. The six femur bones; three PCL/HAp scaffolds and three PEM coated PCL/HAp scaffold were harvested for each several time of 1 week, 2 weeks, 4 weeks and 6 weeks post-implantation. Rats were euthanized and the femur bone was carefully excised, cleaned, and fixed immediately with 4% formaline (24 h at 4°C).

The specimens were taken the radiograph for initial evaluation of the mineralization density within defects. Then the specimens were decalcified with 12% EDTA, pH 7.0, in shaking incubator at 56°C 120 rpm for 20 to 30 days to dissolve calcium from bone. After the decalcification completed, all the specimens were processed of paraffin embedding. Briefly, the sample bones were immersed in running water for 2 h and dehydrated in serial ethanol solutions (70%, 80%, 95% and absolute alcohol), immersed in xylene and embedded in paraffin. The embedded samples were sectioned (7 μm in thickness), and stained with Masson's Trichrome[86]. 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 Image Pro Plus analysis program.

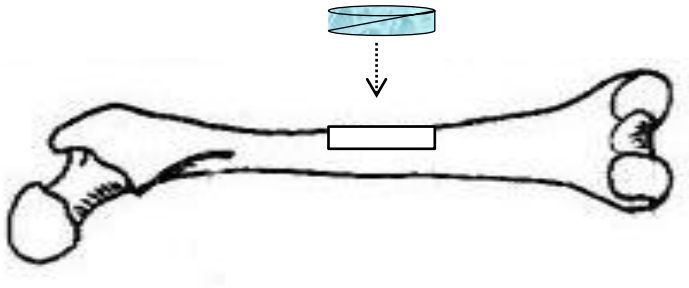


Figure 8 Illustration of the drawing scaffold implanted into rat femur.

Statistical analysis

The descriptive analysis was used in description of the capable of PEM coated PCL/HAp scaffold which improved new bone formation. The t-tests were used in analysis of the data to compare the cell proliferation in MTT assay, cell differentiation in ALP activity, Alizarin red staining of calcium mineraliazation and amount of new bone formation. The probability level less than 0.05 was considered as a statistical significant.

CHAPTER IV

RESULTS

Cell morphology

The morphology of cell attached on PEM coated and non-coated PCL/HAp scaffolds were examined under SEM at 1h, 4h and 16h, as shown in Figure 9(A). The results showed that MC3T3-E1 cells could attach and spread well on all surfaces. After seeding for one hour, the adherent cells appeared round with fine, long filopodia. Cells began to be more elongated when observed at 4 h. At 16h, cells seeded on both glass surface and PEM-coated scaffold showed a better spreading and look more flattened compared to the cells seeded on the non-coated surfaces. It appeared that cells preferred to adhere and attach on the surface of PEM coated scaffold as compared to the non-coated surface. These results suggested the more biocompatible surface of PEM coated scaffold compared to the surface of non-coated scaffold.

Cell proliferation

The results from MTT assays showed the number of viable cell cultured on both coated and non-coated surface of scaffold after seeding for 1 to 5 days were shown in Figure 9(B). The results revealed that the cell number increased when cultured on the scaffold when MTT assay was performed at day 1, 3 and 5 indicated the ability to proliferate on the scaffold surface. However, the number of cells cultured on PSS-co-MA coated PCL/HAp scaffolds was significantly higher than that found of the non-coated PCL/HAp in all times observed ($p \leq 0.05$).

Alkaline Phosphatase (ALP) activity

Alkaline phosphatase activity was measured on cell cultured on both coated and non-coated scaffold surfaces for 5, 10 and 15 days. The result was shown in Figure 10. The activity of alkaline phosphatase was highest at 5 days and then slightly decreased in 10

and 15 days on both surfaces. However, no significant difference on ALP activity was detected in cells seeded on both surfaces at all time detected.

In vitro calcification

MC3T3-E1 cells could differentiate into mature osteoblast when cultured in osteogenic medium that contained ascorbic acid, β-glycerophosphate and dexamethasone [87]. To examine the influence of both coated and non-coated scaffolds on osteogenic differentiation, MC3T3-E1 cells were cultured in osteogenic medium for 21 days. Osteogenic differentiation was judged by the ability of cells to form *in vitro* calcification. The amount of calcium deposition was monitored by Alizarin Red-S staining. The results revealed the higher amount of calcium mineralization on coated scaffolds compared to that on the non-coated scaffold (Figure 10B(a)). The quantitative analysis of calcium deposition was determined by cetylpyritidium chloride destaining (Figure 10B(b)). The results showed a significant greater amount of calcium deposition in cultured on both type of scaffolds compared to the culture on glass surface ($p \leq 0.05$) indicating the potential of PCL scaffold to support osteogenic differentiation, however, no significant difference was observed between the amount of calcium deposition found on both types of scaffold.

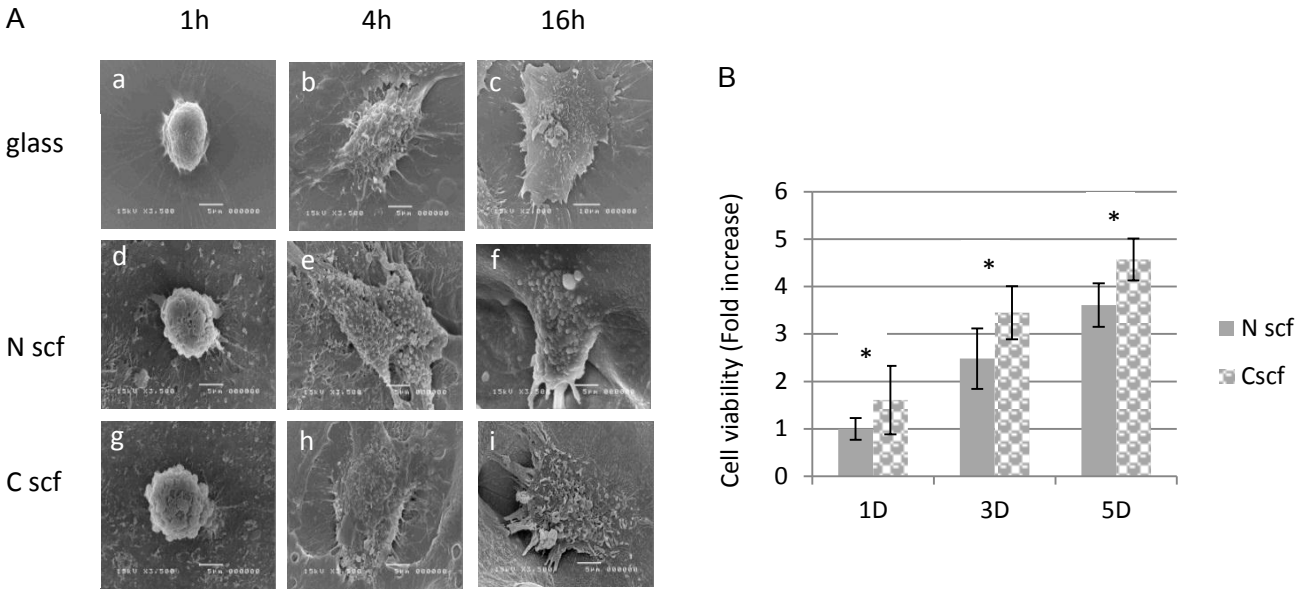


Figure 9 (A) SEM micrograph showed the morphology of MC3T3-E1 cells attached on glass (a, b and c), non-coated scaffolds (d, e and f) and coated scaffolds (g, h and i) after cells seeding for 1, 4 and 16h, respectively. Magnification is 3500x and scale bar is 10μm. (B) Graph showed the data from MTT assay that represented the fold differences ($p \leq 0.05$) in cell viability and proliferation on 1day (1D), 3days (3D) and 5 days (5D).

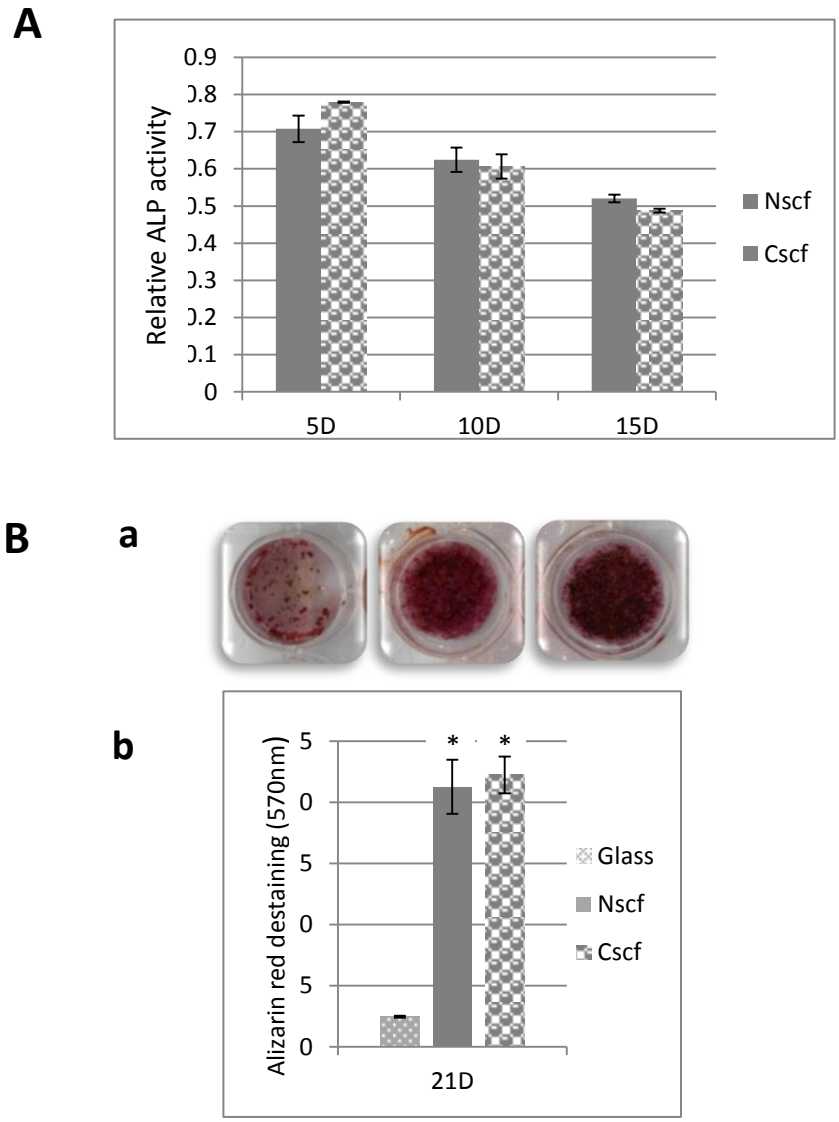


Figure 10 Graph showed the alkaline phosphatase (ALP) activity of MC3T3-E1 cultured on different surfaces. (A) ALP activity after 5, 10 and 15 days on non-coated (Nscf) and coated scaffold surfaces (Cscf). (B). (a) Photograph of the 21 days cultures stained with Alizarin red-S to monitor the level of *in vitro* calcification. (b) Graph showed the quantitative analysis of calcium deposition eluted by cetylpyritidium chloride and measured by spectrophotometer (570nm). Amount of calcium depositon on both of scaffold surface was significantly higher than glass surface and significantly compared in coated and non-coated scaffold surfaces.

Animal test

Scaffolds were implanted in rat femurs. The radiographic examination was performed since it is the simple way and non-invasive to evaluate mineral deposition [40, 88]. The radiographic images, as shown in Figure 11, suggested the gradually increased of mineral within the circular defects from 1st to 6th week. However, the density of scaffold in all samples was less dense than the surrounding bone. At first week, the circular defect could be obviously traced. There was no difference observed on the density of scaffold when compared between the group of coated and non-coated scaffold implanted. In the 2nd week, some radiopaque clusters of mineralized tissue within circular defect were observed. At 6th week, both groups had a density closed to the surrounding bone and revealed high density of new bone formation within coated scaffold implantation more than surrounding bone.

The histological sections of femoral bone after scaffold implantation for 1, 2, 4 and 6 weeks were shown in Figure 12. The sections were stained with Masson's Trichrome to demonstrate osteon, and cellular detail [40, 89]. Histological analysis revealed the new bone formation close to the marrow surface of the scaffold and periphery cortex. Histomorphometric analysis indicated the higher amount of new bone formation within and around the coated scaffolds compared to those found in the non-coated scaffold in all time detected. At 1st week, both coated and non-coated scaffold was filled with loose connective tissue developed from marrow side of scaffold. No sign of inflammation was observed in both groups. In the 2nd week, more woven bone formation was observed within the coated scaffolds compared to the non-coated one. The new bone formation appeared as trabeculae bone with lacunae. The direction of trabeculae bone growth was found from the marrow surface towards the cortex. At 4 weeks, the amounts of bone formation between two groups were comparable, however, the new direction of bone formation from the cortical towards the defected area was observed. Finally at 6 weeks in cross section defect, the cortex bone was found covered the defected area.

Histomorphometric analysis of new bone formation was performed using image analysis software (Image-Pro Plus) and the results were shown in figure 12B. The results revealed the increase amount of new bone formation start from the1st week in both coated and non-coated scaffold (7.81±4.00% and 2.95±0.73%). Statistical analysis revealed the significantly increased of new bone formation from the 2nd to the 6th week in the group of coated scaffold ($p<0.05$). The amount of new bone formation from the 2nd to 6th week in the coated scaffold was 37.61±12.96%, 44.11±7.94% and 51.26±6.58%, respectively when compared with 10.86±2.31%, 22.54±0.81% and 38.99±1.17%, respectively in the non-coated scaffolds.

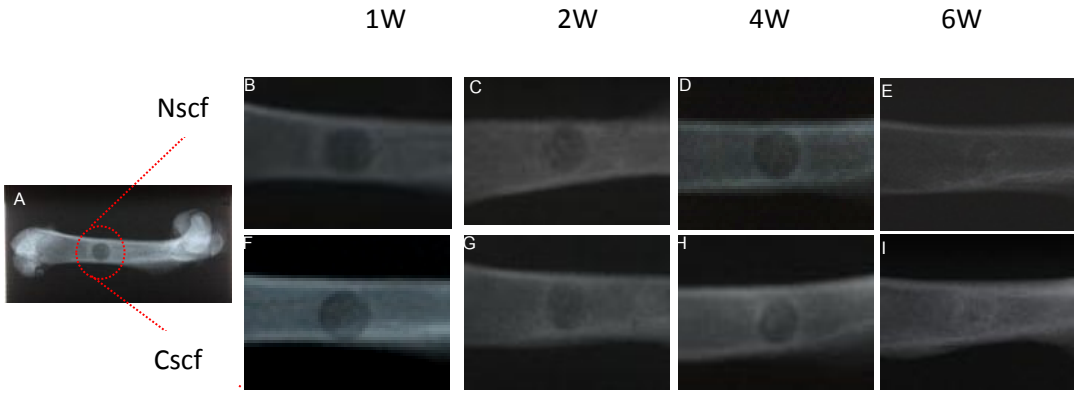


Figure 11 X-ray films of circular defects in femoral bone (A) post operation 1(B and F), 2(C and G), 4(D and H) and 6(E and I) weeks. The x-ray examination showed no different explicitly between non-coated (B, C and D) and coated scaffolds (E, F and G) at 1 to 4 weeks. The density of new bone formation within scaffold dramatically increased from the 1st to 4th week and revealed the high density of bone formation at the 6th week in coated scaffold implantation.

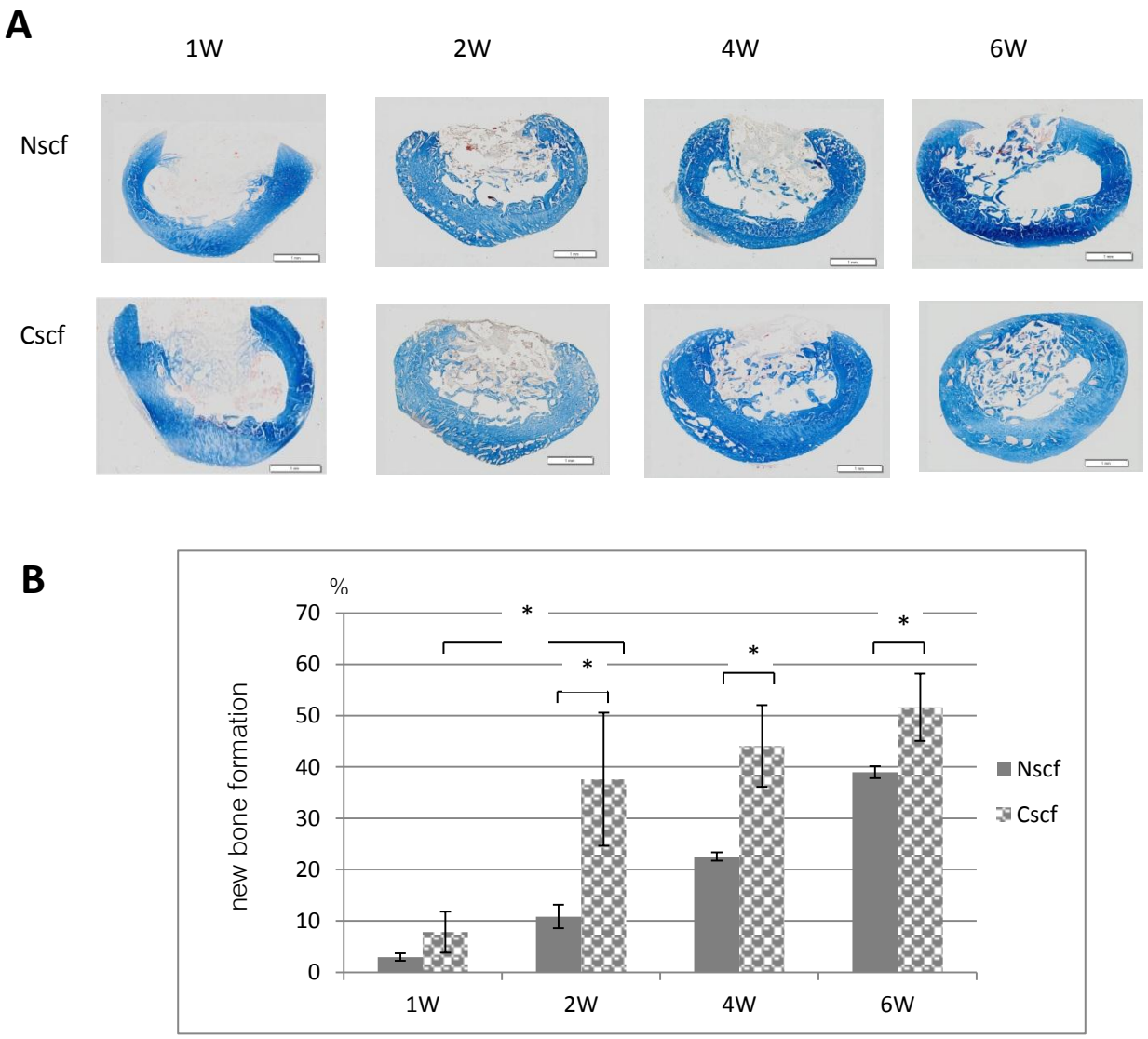


Figure 12 Histological illustration demonstrated the in vivo bone formation of coated and non-coated scaffold. (A) Photograph from histological analysis showed the new bone formation after implanted with coated (Cscf) and non-coated scaffolds (Nscf) in circular defect for 1 (1w), 2 (2w), 4 (4w) and 6 weeks (6w) implantation in rat femoral bone. The cross sections were stained with Masson's Trichrome. (B) Graph showed the percentage of new bone found within the coated and non-coated scaffolds slightly increased from 1w to 6w. In Cscf group, the amount of new bone formation was significantly increased between 1w and 2w. At 2w and 6w, the percentage of new bone formation in Csf group was significantly higher than Nscf group. * represented the significant difference ($p \leq 0.05$).

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

The development of PCL/HAp scaffold to be used as bone tissue engineering had been in attention of several researches [26, 45, 90, 91] due to the good biocompatibility and controllable biodegradability of polymers which took together with the advantage of hydroxyapatite particle. From the previous studied, fabrication of PCL scaffold incorporated hydroxyapatite by particular leaching technique, a simple and effective method, with proper pore size and mechanical properties combined with alkaline treatment to improved the water absorption ability was reported. This PCL/HAp scaffold can serve a new bone formation both *in vitro* and *in vivo* experiments [4, 26]. However, modification of scaffold to develop better properties for bone tissue engineering is still needed. For example, Azevedo MC et al. improved the PCL/Hap scaffold properties to obtain the high modulus of composite scaffold by increasing the fillers [45]. By the technique of fabrication, Jessica M Williams et al. modified the scaffold used a selectived laser sintering [26] while Frank R and coworker fabricated by Electrospun PCL/PLA/HA based nanofibers [92]. In this study, coating surface of biomaterial had been done to develop the surface property of PCL scaffold.

PEM surface coating had been selected because of its uncomplicated procedure and effective. These advantages make this technique attracted an interest in field of biomaterial and tissue engineering. Moreover, PEM can be applied to coat variety of bulk materials such as polymer, ceramic and metal. PEM coating with PDADMAC/PSS/PSS-co-MA was first reported by Angwarawong and coworkers who showed that surface modulation with strong polyelectrolyte multilayer could support adhesion and differentiation of MC3T3-E1 and primary human osteoblast cell as well as the in vitro calcification[8].

PSS-co-MA is a copolymer which contains strong sulfonate group of PSS to form electrostatic linkages and enhance film stability and weak carboxylic group of maleic group. Since maleic acid has two ion-exchangeable sites and exhibits the lower water uptake probability than that of the sulfonic acid group[93], the introduction of maleic acid group into PSS can increase the membrane charge density and prevent excessive swelling. Reported from Uragami et al.[94] prepared poly(styrene sulfonic acid) (PSSA)/poly(vinyl alcohol) (PVA) blend membrane to investigate the active and selective transport of alkali metal ions such as potassium ion in diffusion dialysis using pH difference as the driving force. Moon-Sung Kang et al and C.W. Lin et al. using PSS-co-MA with poly(vinyl alcohol) (PVA) as PVA/PSS-co-MA crosslink membranes to exhibited low electrical resistance and highly swelling property for applied to dialytic membrane processe[93, 95].

The PSS-co-MA PEM coating surface on PCL/HAp scaffold would combined the benefit of the PEM coating effect with good properties of PCL/HAp material. First benefit was the advantages of tissue reaction on PSS-co-MA surface. The second benefit was from the effectiveness of bulk material of PCL/HAp scaffold. In tissue engineering, cell adhesion is closely related to the surface properties of biomaterials[96]. It is commonly accepted that the adhesion of cells to solid substrate is influenced by several substratum surface properties as surface charge, roughness and topography[6]. Since surface modification only changes the outermost surface composition of a biomaterial, its bulk properties do not change. In addition, surface modification can provide accessible and chemical functional groups for the immobilization of drugs, enzymes, antibodies or other biologically active species for a variety of biomedical applications.

The ability of PSS-co-MA PEM surface coated on 3D scaffold material demonstrated the better support osteoblast behavior both *in vitro* study and new bone formation *in vivo* study. In SEM study, MC3T3-E1 cells could spread well on all PCL/HAp scaffold surfaces, implied the biocompatibility of both PCL/HAp surface and PSS-co-MA

surface. At the 4 h cells on PSS-co-MA surface showed a better spread compared to the non-coated but at 16 h, no difference between two PCL surface was not observed. The results were in agreement with Anwarawong study who reported that MC3T3-E1 cells could attached and growth not different on glass and PSS-co-MA surface at 4 and 16 h. This characteristic of cell spreading well at 4h on PSs-co-MA scaffold surface may be caused the characteristic of cell spreading on 3D surfaces were elongated but smaller compared with 2D surface of glass which more spread and flatten agree with the study of Dutta et al.[97].

Cell proliferation and differentiation strongly depend on the chemical and physical properties of the surface of a tissue engineering scaffold[98]. In this study, cell cultured on PSS-co-MA coated scaffold could enhance cell proliferation, based on MTT assays which based on the ability of viable cells to reduce a tetrazoluim-salt to purplish formazan product by dehydrogenase enzyme from cell mitochondria. The results showed the continuous cell proliferation from day 1 to day 5 as shown by the increasing value of relative MTT absorption. Because of the much better support from cell adhesion in the initial stage, the cultured cells on PSS-co-MA surface can rapidly proliferation. This study agreement with the results in previous study that found the PSS-co-MA coated on glass surface could support cell proliferation at 5D

In general, the process of osteoblast differentiation can be characterized into at least three stages:(a) cell proliferation, (b) matrix maturation, and (c) matrix mineralization[58]. *In vitro* matrix maturation and mineralization are usually enhanced by addition of specific osteogenic inducers. During proliferation (a), several extracellular matrix proteins (procollagen I, TGF-β, and fibronectin) can be detected. The matrix maturation phase (b) is characterized by maximal expression of alkaline phosphatase (AP). Finally, once mineralization is completed, calcium deposition can be visualized using adequate staining methods. When the cultured cells attached and proliferated on the suitable

environment, the cells on such scaffolds were differentiated and entered into the matrix maturation phase rapidly[58, 99]. ALP activity of cells the cultured for 10 and 15 D on scaffold surface slightly decreased, implied that the cultured cell both coated and non-coated PCL/HAp scaffold were going into the mineralization phase. These possibilities might explain the reduction of ALP activity after the 5th day in culture. When the cultures were maintained for 21 days, a greater amount of calcium deposition on PSS-co-MA coated surface scaffold was found as judged by calcium staining that could be quantitated by destaining and subjected to spectrophotometical analysis. More staining of calcified nodule formation on PSS-co-MA coated scaffold surfaces could reflected the advanced differentiation and calcification onto surface of cells. These results supported the potential of PSS-co-MA coating on scaffold in osteogenic differentiation. The better supportive results may derived from the superior propertied of bulk material of PCL/HAp as scaffold since HAp particles may dissolve to release calcium and phosphate ions. These released ions had been demonstrated to be able to conditioned the environment and enhance bone cell mineralization[5, 15].

In vivo study also indicated the ability of PSS-co-MA PEM coating on supporting osteoblast differentiation. The amount of bone formation within the first two weeks implantation with coated scaffold was comparable to the amount found with non-coated scaffold. However, the better formation of bone could be observed after two weeks and within 6th week implantation, PEMs coated scaffold could support the completed healing of the defect. It's has been reported that the process of new bone formation not only involved in suitable material framework, but also related to surfaces which play an important role in a biological system. Most of biological reaction occurred at the surface and surface interfaces[6] which noticed the formation of connective tissue around coated scaffold faster that the control in the histological staining. The effect of surface modification effect to bone defect repair had studied extensively[3, 100].

Comparing to others modification methods, such as; the use of stem cell, coating with plasma rich platelet[40] or combine growth factor with the scaffold[101], the PEM dipping modified surface was more practical and economical. The electrostatic multilayer technique had been applied to other applications as well, such as Ti implant, in order to induce bone integration[102, 103]. All of the results in coated scaffolds demonstrated the effect of PSS-co-MA PEM coating technique could be support new bone formation. However, it is still a question whether the modification of surface in a nanoscale level by PEM might provide the suitable environment that could enhance the rapid bone formation[104]

Since surface modification only changes the outermost surface composition of a biomaterial, no change was observed in regards of the bulk properties. In addition, surface modification can provide accessible and chemical functional groups for a variety of biomedical applications. Cell attachment and proliferation strongly depend on the chemical and physical properties of the surface of a scaffold[40, 105, 106]. Many researchers prepared various specific surfaces in order to study the correlation between surface topography and cell attachment and migration [107-109]. As the study of David S Salloum clearly detect the protein peaks as compared to the thick negatively charged surface, believe that electrostatic interactions play a major role in the adsorption process [68]. In other studies, sulfonated surfaces have been prepared by surface chemical reactions on polymers in order to produce a similar protein repellent effect. Comparing different surfaces with different ionic charges, sulfonated surfaces had shown a higher affinity property for proteins. Even when comparing the surfaces containing different sulfonation levels, higher sulfonation could led to more fibrinogen adsorption, whereas the higher negative charges were expected to do the opposite role. However, the mechanism is not yet understood[110,

111]. More studies are needed to study the mechanism of protein adsorption onto ionic surfaces[68]. Surface modifications of polymers to induce a surface charge have been used, mainly to introduce a net positive or negative surface charge that will selectively adsorb proteins of opposite charge. This protein absorption may be correlated the attraction of protein and growth factor in healing process of bone.

Conclusion

The ability of PEM surface modification on PCL/HAp scaffold was demonstrated. Biological testing showed that the PSS-co-MA coating could support new bone formation both *in vitro* and *in vivo*, despite not exactly reveal *in vitro*. However *in vivo* study showed the advantage of this surface coating in improvement of new bone formation in the defect. The success of PEM coating PCL/HAp scaffold need further study to continuous development for applied in clinical trial.

This study showed ability of development of PEM coating PCL/HAp scaffold to be one choice for bone tissue engineering.

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