CHAPTER II
LITERATURE REVIEW

2.1 Bone

Bone is a major part in a human skeleton, which is a specialized type of connective tissue. Bone functions to provide structural support for the body, protect vitals, and serve as calcium, phosphate and others ions' reservoir in the body. Bone is composed of extracellular matrix and three types of cell: osteoblasts, which synthesize the organic components of the matrix; osteocytes, which are found in cavities within the matrix; and osteoclasts, which are multinucleated giants cells involved in the resorption and remodeling of bone tissue (Junqueira *et al.*, 2003).

2.1.1 Bone Cells

Bone cells are classified into four major types:

2.1.1.1 Osteoprogenitor Cells

Osteoprogenitor cells are spindle-shaped cells, which are derived from embryonic mesenchyme. Osteoprogenitor cells are capable of differentiating into osteoblasts (Gartner *et al.*, 1993).

2.1.1.2 Osteoblasts

Osteoblasts are located at the surface of bone tissue, derived from osteoprotogentior cells. Osteoblasts are responsible for synthesis of the organic components of bone matrix which are type I collagen, proteoglycan, and glycoproteins. The matrix components are secreted at the cell surface, which is in contact with older bone matrix, During this process, a layer of new matrix between the osteoblasts layer and the previously formed bone is produced, called osteoid. Osteoid consists of type I collagen and noncollagenous proteins. This process, bone apposition, is completely deposit by calcium salts into the newly formed matrix (Junqueira *et al.*, 2003). Some osteoblasts are gradually surrounded by newly formed matrix and become osteocytes. During this process, a space called a lacuna is formed. Osteoblasts become entrapped in lacunae but maintain contact with other

cells via their cytoplasmic processes. Once this happens, the cells are known as osteocytes.

2.1.1.3 Osteocytes

Osteocytes, which derived from osteoblasts, are mature bone cells housed in the lacunae. There is only one osteocyte found in each lacuna. Osteocytes have narrow cytoplasmic processes extending through canaliculi in the calcified matrix. Processes of adjacent cells make contact via gap junctions, and molecules are passed via these structures from cell to cell. Osteocytes are nourished and maintained by nutrients and metabolites within canaliculi. The flat, almond-shaped osteocytes exhibit a significant reduced rER and Golgi complex and more condensed nuclear chromatin in comparison with osteoblasts. Osteocytes are actively involved in the maintenance of the bony matrix, and their death is followed by resorption of this matrix (Junqueira *et al.*, 2003).

2.1.1.4 Osteoclasts

Osteoclasts are very large, motile, and multinucleated cells *(Gartner et al., 1993)* that are derived from fusion of monocytes. Osteoclasts play a role in bone resorption. Bone resorption takes place when osteoclasts secrete acid (acid phosphatase)that creating an acidic environment and decalcifying the surface layer of bone followed by secretion of acid hydrolase, collagenase, and other proteolytic enzymes that degrade the organic portion of the bone. Finally, osteoclasts resorb the organic and inorganic residues of the bone matrix (Gartner *et al.*, 1993).

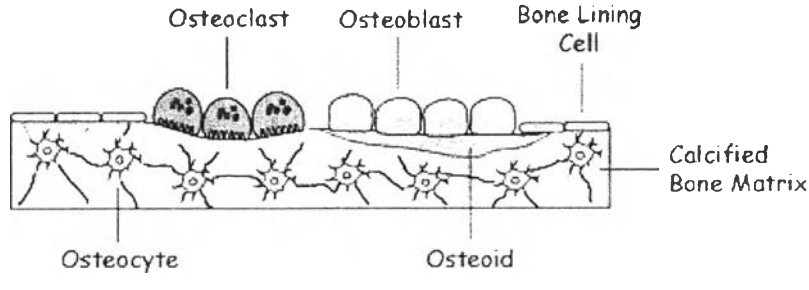


Figure 2.1 The major types of bone cell (http://www.iofbonehealth.org.html).

2.1.2 Some Types of Osteoblast-like Cells

2.1.2.1 SaOS2

The SaOS2, human osteosarcoma, cell line established from the primary osteogenic sarcoma of an 11-year-old Caucasian woman in 1973 (ATCC HTB 85). The SaOS2 cell line expresses a more limited number of osteoblast phenotypic markers when compared to MG63, another human osteosarcoma cell type, in which MG63 cell line can expresses type I collagen, ALP, osteopontin, bone sialoprotein, and osteocalcin (OC calcitriol dependant) (Bilezikien *et al.*, 2002).

2.1.2.2 MC3T3-E1

The MC3T3-E1 cell line (mouse calvaria-derived, pre-osteoblastic cells, ATCC-CRL 2593) is a spontaneously immortalized cell line selected by the 3T3 passaging protocol. MC3T3-E1 cells behave as immature, committed osteoblasts, which go to differentiate in response to intracellular and extracellular cues. Upon reaching confluence, this clonal cell line differentiates along the osteoblast lineage, sequentially expressing characteristic osteoblast phenotypic markers including type I collagen, ALP, osteopontin, osteonectin, bone sialoprotein, osteocalcin, and matrix metalloproteinase1 (MMP1) (Bilezikien *et al.*, 2002).

2.1.2.3 hFOB

The hFOB cell line is a clonal, conditionally immortalized human fetal cell line capable of osteoblastic differentiation and bone formation. Cells proliferate as if immortalized at 33.5°C but differentiate at 39.5°C. Cells grew at a temperature of 33.5°C with rapid cell division (doubling time of 36 hrs), whereas little cell division occurs at a restrictive temperature of 39.5°C (doubling time of 96 hrs). hFOB cells show normal bone cell characteristics with expression of type I collagen, bone morphogenic protein (BMP), ALP, osteopontin, osteonectin, bone sialoprotein, and osteocalcin (Harris *et al.*, 1997).

2.1.3 Bone Matrix

Extracellular matrix (ECM) (Fig. 2) is the part of the body, functions to maintain the body in an appropriate form and shape. There are two major substances in ECM:

2.1.3.1 *Organic Substances* (ca. 25% of ECM)

Organic substances consists of collageneous proteins and noncollageneous proteins (i.e. type I collagen, glycoprotein, osteopontin, osteocalcin and proteoglycans).

2.1.3.2 *Inorganic Substances* (ca. 70% of ECM)

Inorganic substances consists of complex calcium and phosphate (hydroxyapatite).

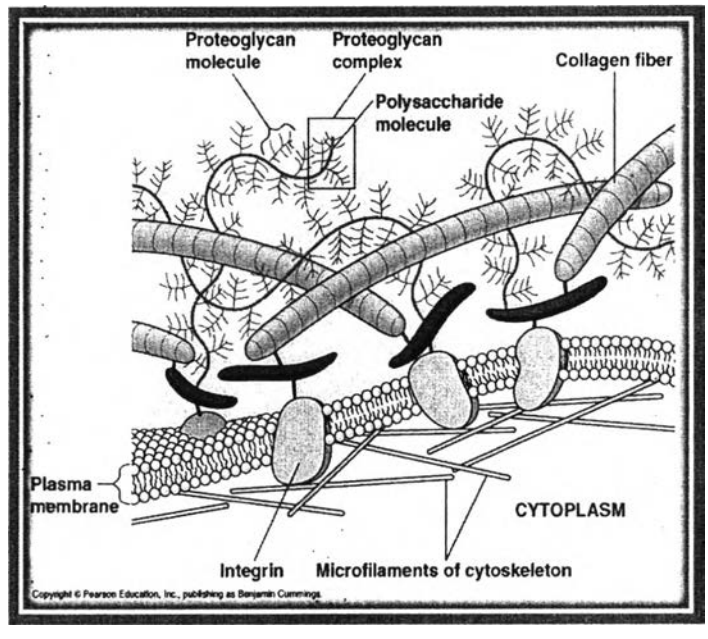


Figure 2.2 The extracellular matrix.

2.1.4 Bone formation

Bone formation is occurred in ordered step. Firstly, recruitment and proliferation of osteogenetor cells occurred from surrounding tissue. Secondly, osteoblastic differentiate. Followed by matrix formation and finally mineralization. (Shin *et al.*, 2003)

2.1.4.1 *Process of bone remodeling*

The cycle where new bone resorption and formation occurred, called bone remodeling. At the beginning, blood carries osteoclasts (bone-resorbing cells) to the bone, and then they attach themselves to the bone, releasing acids and

enzymes which are resorb the older bone structure. After the osteoclasts resorb the bone, they disappear, then osteoblasts(bone-forming cells) appear instead and coat the resorbed area with adhesive substances and produce bone proteins such as collagen to help calcium adhere to the bone proteins. After this stage is completed, new bone is forms. (Fig. 3)

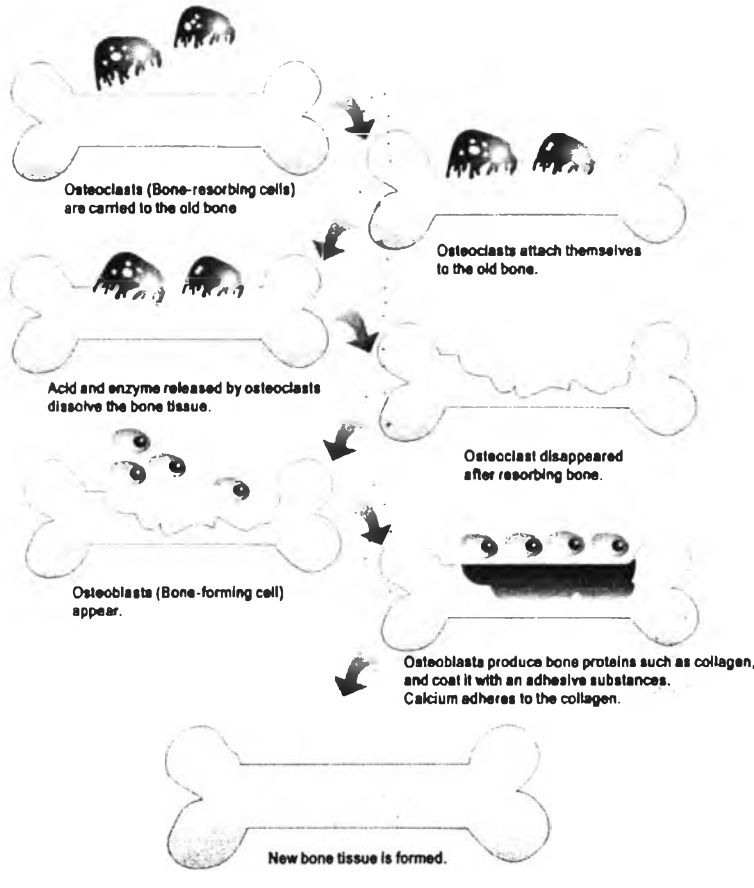


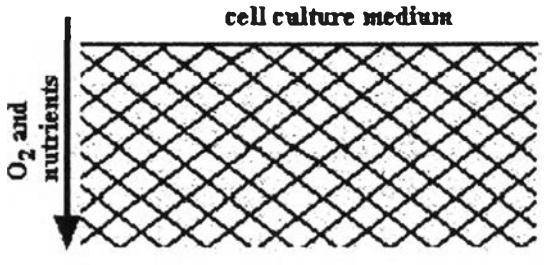
Figure 2.3 Bone Remodeling Cycle.

2.2 Bone Tissue Engineering

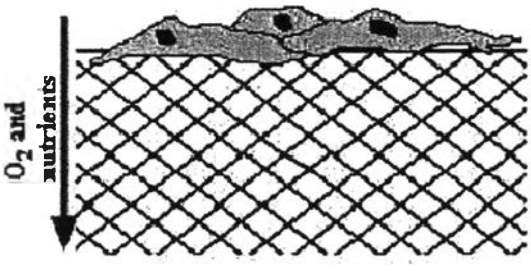
Nowadays, bone failure, bone injury or bone fracture is the major health problem in human. Organ or tissue transplantation is such a suitable therapy; however, it still has limitation in resources shortage, infected problem, immune rejection and so on.

Bone tissue engineering is a technique applying the principles between life science and material engineering, purposing to create artificial tissue or organs to direct tissue regeneration (Shin *et al.*, 2003). Hutmacher (2000) classified tissue engineering program into six phases:

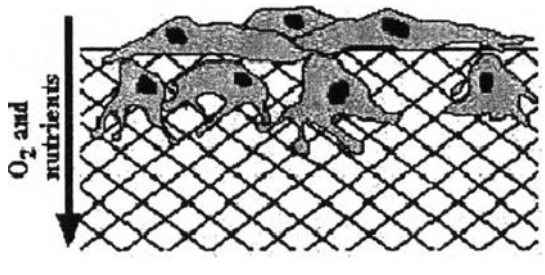
I. Fabrication of bioresorbable scaffolod
II. Seeding of the osteoblast/chondocytes populations into the polymeric scaffold in a petri dish
III. Growth of premature tissue in a dynamic environment (spinner flask)
IV. Growth of mature tissue in a physiologic environment
V. Surgical transplantation
VI. Tissue-engineered transplant assimilation/remodeling



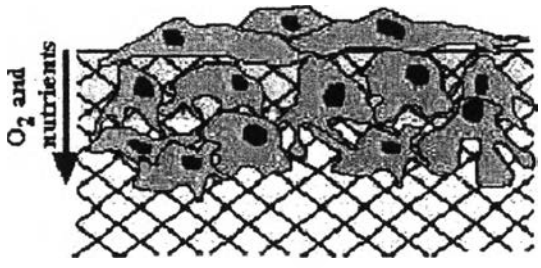
a) Tissue engineering scaffold which contain pores structure. Oxygen and nutrients are supplied from liquid cell culture medium.



b) Cell seeded on scaffold.



c) Cells start to proliferate and migrate into the pores of scaffold.



d) The cells completely place in the pores and start to lay down their extracellular matrix

Figure 2.4 Concept of cell growth on the scaffold. (Sachlos *et al.*, 2003)

2.3 Biomaterials Applied for Tissue Engineering

In order to achieve successful regeneration of damaged tissue or organs, several materials have been considered including synthetic polymers (Shin *et al.*, 2003). Recently, the synthetic polymers play an important role in tissue engineering. Biodegradable polyesters, such as poly(caprolactone) (PCL), poly(lactide) (PLA), and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) are widely used for medical applications because of their biodegradability, biocompatibility, and bioresorbability (Bajgai *et al.*, 2008; Chung *et al.*, 2007).

2.3.1 Poly(caprolactone) (PCL)

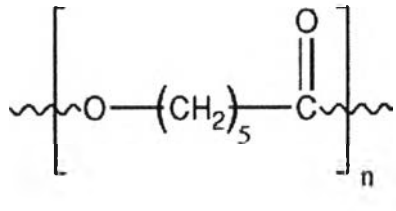


Figure 2.5 Chemical structure of Poly (caprolactone) (PCL).

Poly(caprolactone) (PCL) (**Fig. 2.5**) can be prepared by ring opening polymeriazation of ε-caprolactone. PCL is a semicrystalline polymer with a melting temperature (T_m) of ca. 60 C and glass temperature (Tg) of ca. -60 C (Mattanavee *et al.*, 2009).

Due to the presence of ester linkages in the PCL backbone, allowing hydrolytic degradation of PCL. The degradation products, carbon dioxide and water, are endogenous compounds and they are non-toxic. PCL, therefore is concerned to be used for medical approaches, such as drug carriers, engineered skin, and scaffolds for supporting the growth of cells.

2.4 Surface Modification

Surface modification technique have been developed to improve wettability, adhesion and printing of polymer surfaces by introducing polar groups (Goddard *et al.*, 2007).

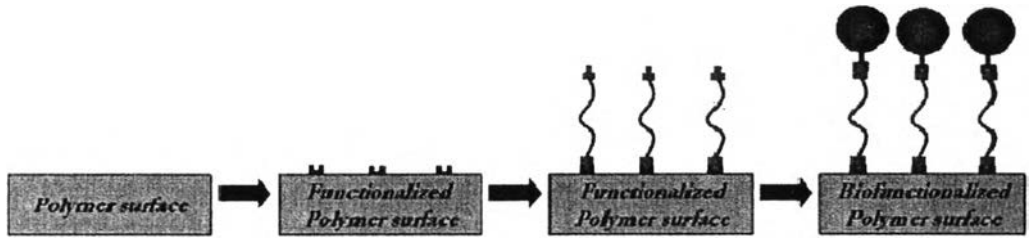


Figure 2.6 Concept of biological surface modification.

Polyesters are biocompatibility and biodegradability; however, they also have some limitations. The major limitation is their hydrophobicity which may interupt cell adhesion and cell growth (Chung *et al.*, 2007).

Techniques for surface modification can be classified in two major classes: non-permanent (non-covalent attachment of functional group) and permanent (covalent attachment). In this project, we focus on permanent surface modification's techniques:

2.4.1 Plasma treatment

Plasma treatment is the way to introduce proper functional grous on the surface of the polymer only by selecting and applying some suitable gas.

2.4.2 Hydrolysis

Hydrolysis or alkaline treatment (Fig. 2.7) is another simple technique to create reactive functional groups such as; carboxylic acids (-COOH), and hydroxyls (-OH).

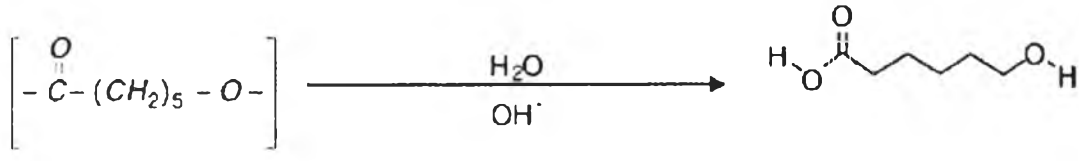


Figure 2.7 Poly(caprolactone) undergoing hydrolysis of its ester linkages.

Yang *et al.*,(2001) hydrolyzed PLA-surfaces by treating with 0.25 M NaOH/ethanol for improving its hydrophilicity. They showed that the low concentration of alkaline solution was added due to preventing bulk degradation.

2.4.3 Aminolysis

Aminolysis is a chemical reaction between ester groups and amino groups, resulting in amide linkage. Mostly, 1,6-hexanediamine has been used for aminolysis and followed by conjugated with macromolecules like collagen or gelatin.

Jacorkar *et al.*,(2007) introduced amine group on PLA film surface by photoinduced grafting. Their result showed the improvement of MC3T3 fibroblast attachment.

2.5 Immobilization of Biomolecules onto Polyester Surface

Since most of polyesters are lack of chemical functionalities, it is normally difficult to be preferable for protein interaction, the covalent binding. The method of aminolysis between diamine and polyester matrix introduces functional amino groups, through which proteins such as gelatin, laminin, chitosan, Arg-Gly-Asp (RGD)-containing peptide, collagen etc., can be further attach on the surface of polymeric scaffolds.

Four major protein immobilization techniques are currently used: (i) covalent binding, which attachment of the active component to the substrate surface uses as chemical reaction to the activated surface groups (Wang *et al.*, 2003; Yang *et al.*, 2003); (ii) entrapment, which is a physical reaction of the active components into a film, a gel or coating (Cui *et al.*, 2003); (iii) crosslinking, which combines features of both covalent binding and entrapment in which crosslinking agents are used both to polymerize a base layer or film and to anchor the entrapped protein molecules by forming intermolecular linkages between the substrate and the protein molecules (Karakecili *et al.*, 2008); and (iv) adsorption, which applies a protein solution to a substrate or film and allows the molecule to adsorp to the substrate over a specified time period (Woo *et al.*, 2007). The example of surface modification procedures are followed:

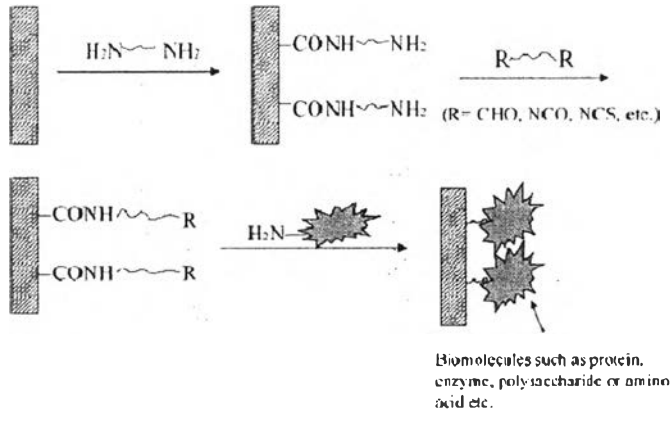


Figure 2.8 Aminolysis and further immobilization of biomolecule on PCL Membrane. (Zhu *et al.*, 2002)

2.5.1 Biomolecules

2.5.1.1 Bovine Serum Albumin

Bovine serum albumin is a large globular protein (66,000 Dal) with a good essential amino profile. BSA is an acidic protein with isoelectric point of 4.2-4.8 is well known to mainly adsorb on the Ca-sites on HAp by ionic interaction between carboxyl acid groups on BSA and the positively charged Ca-sites. (Boonsongrit, Yaowalak *et al.*, 2008).

2.5.1.2 Crude bone protein from pork bone

Bone is physically a hard tissue abundant with mineralized extracellular matrix which makes up about 90% of the total weight of compact bone. Bone extracellular matrix comprises 60% of the microcrystalline calcium phosphate resembling hydroxyapatite, 27% of fibrillar type I collagen and 3% of the minor collagen types and other bone proteins including osteocalcin, osteonectin, phosphoproteins, sialoproteins and glycoproteins, as well as proteoglycans, glycoaminoglycans, and lipids (Hauschka *et al.*, 1986).

Bone extracellular matrix apparently is a depository of bioactive proteins and peptides. In healthy tissue, bone extracellular matrix controls the presentation and distribution of growth factors accurately for any specific condition (Hauschka *et al.*, 1986). Bioactivities of the existent growth factors in bone extracellular matrix retain even in the demineralized condition. In the mid-60s, Urist (Urist, 1965) demonstrated the induction of new ectopic bone formation from a decalcified bone matrix and proposed the existence of osteoinductive molecules in the matrix that direct differentiation of precursor cells into bone forming osteoblasts. The study of Somerman et al. in 1983 (Somerman *et al.*, 1983) confirmed the proposition. They reported that demineratized bone matrix extracted with 0.05 M Tris HCl, pH 7.2 containing 4 M guanidine HCL contained all the information necessary for bone formation. The extracts illustrated chemotactic activity to osteoblast-like cells and itself was heat-labile and sensitive to trypsin, which therefore is a protein-like material. The deminearalized bone powders induced new bone formation when implanted subcutaneously.

Bone extracts form the demineralized bovine bone powder was investigated the growth factors in a quantitative fashion by Hauschka et al. in 1986 (Hauschka *et al.*, 1986). The result illustrated abundant growth factor activity as high as 570 GFU/g of dry bone (200 pg protein/GFU) in the extracts obtained by 0.5 M EDTA, 0.05 M Tris-HCl, pH 7.5 extractions. The isolated crude bone extracts and growth factors significantly stimulated mitogenic activity on osteoblast of the newborn rat calvaria while depressing alkaline phosphatase specific activity by 2-3 folds.

Extracts from bone contain a lot of factors for chemotaxis, attachment, proliferation and differentiation of bone cells. In 2000, Hou LT *et al.* (Hou *et al.*, 2000) studied the effects of bone extracts on behaviors of human periodontal fibroblasts in vitro and revealed that the periodontal fibroblasts cultured with bone extracts possess osteoblastic characteristics by presenting genetic expression of alkaline phosphatase, osteocalcin, osteonectin, sialoproteins and bone morphogenetic protein-7 and were also capable of forming mineralized foci in vitro. The results corresponded to those of the previous studies which also demonstrated that extracts of bone matrix regulate osteoblast function (Syftestad and Caplan, 1984; Cho *et al.*, 1992).

Crude bone extracts, therefore, evidently encompasses enormous active proteins and growth factors which facilitates new bone formation. Extraction of the crude bone protein with the intricate procedures seems not to obliterate bioactivities of those factors. These presumptions initiate the plan to exploit crude proteins extracted from demineralized bone in regenerating bone tissue in this thesis.

2.6 Literature Reviews

Yang *et al.*, (2001) examined the adhesion, spreading, growth, differentiation, and phenotypic modulation of human osteoprogenitors on surface-modified two-dimensional PLA films. The surface was modified with either fibronectin or the cell adhesion motif RGD. The study demonstrated that there are poor cell adhesion and growth on unmodified PLA films whereas fibronectin or RGD modified PLA films can enhance cell adhesion, spreading and differentiation.

Zhu *et al.*, (2002a) introduced amino groups onto poly(caprolactone) (PCL) surface by reaction between 1,6 hexanediamine and the ester groups of PCL. Then biomacromolucule such as gelatin, chitosan or collagen were further immobilized on the aminolyzed PCL. The results showed that endothelial cell cultured on immobilized amonolyed PCL was improved because of its high cell attachment and cell proliferation.

In the same year, Zhu *et al.*, (2002b) also studied chitosan immobilized PLA film surface using the photosensitive hetero-bifunctional crosslinking reagent, 4-azidobenzoic acid. Heparin was used to modify chitosan molecules ommobilized PLA forming a polyelectrolyte complex on PLA surface. Cell culture assay indicated that PLA surface with CS/Hp complex showed echances cell adhesion.

Tiaw *et al.*, (2003) modified the surface of PCL films by using femtosecond laser and excimer laser technique. The study demonstrated that laser surface modification on the PCL can be achieve with the high degree of success and precision. Both laser techniques can enhance the wettability of the hydrophobic of PCL surface.

They also referred to the experiment from Schantz *et al.*, who treated PCL membrane with NaOH solution using osteoblast-like cells to study for cell attachment and cell proliferation. The result indicated that NaOH treated PCL can enhance both cell attachment and cell proliferation of osteoblast-like cells.

Cheng *et al.*, (2004) prepared PCL film by using solvent casting technique and biaxial stretching technique. The PCL film was pretreated by using Argon plasma, and UV polymerized with acrylic acid. Then immobilized with collagen, resulting in improving the hydrophilicity of modified PCL film observed by water contact angle.

Zhu *et al.*, (2006) synthesized poly (L-lactide-co-caprolactone) (PLLC) copolymer. HMD was introduced on the surface of PLLC to provide amino groups on its surface. After that either fibronectin or collagen was immobilized by using alutaraldehyde as a coupling agent. The results demonstrated that protein-immobilized PLLC got more hydrophilic and more homogeneous cell cultural. They concluded that the modified PLLC, compare to unmodified PLLC, promote more preferable to tissue regeneration.

Santiago *et al.*, (2006) prepared PCL disks with an average thickness about 227.7 $\pm$ 16.3 μm and then added 1,6 hexanediamine to modified their surface. Peptide sequences derived from ECM protein laminin were immobilized onto the PCL surface using carboiimide as a coupling agent. A high number of cell can be observed in treated PCL disk compared with untreated PCL. This study indicated that

peptide sequences-immobilized PCL can promote the attachment between cells and polymer scaffold.

Savarino *et al.,* (2007) studied the ability of cellular construction of PCL porous scaffold, seeded with autologous rabbit bone marrow stromal cells (BMSCs) and bone morphogenetic protein 4-expressing BMSCs. The results showed that PCL seed with growth factor expressed cell stimulated new tissue formation.

Pompe *et al.,* (2008) used thin films of P(3HB) and P(3HB-4HB) containing 10.8% 4HB which were modified by three different methods : (i) alkaline hydrolysis; (ii) H_2O plasma; and (iii) NH_3 plasma treatment. The outcome of their study shown that all treatments increased the hydrophilic of the polymer samples, which caused from newly created amine or carboxylic functionalities for NH_3 plasma and H_2O plasma treatments, respectively, and ester hydrolysis for treatments with alkaline aqueous solutions.

Mattanavee *et al.*, (2009) showed the aminolyzed polycaprolactone (PCL) film or electrospun fiber ma, using N,N'-disuccinimidylcarbonate as a coupling agent. Followed by immobilized with biomolecules such as collagen, chitosan and Gly-Arg-Gly-Asp-Ser (GRGDS) peptide in the water. Three different cell lines, e.g. mouse calvaria-derived preosteoblastic cells (MC3T3), mouse fibroblasts (L929), and human epidermal keratinocytes (HEK001) were cultured to evaluate the potential for using PCL as a scaffold. The existence of NH_2 groups on polyester surface was characterized by ninhydrin or fluorescamine analysis. The paper was shown that NH_2 density increased with 1,6-hexanediamine concentration. Mattanavee et al also found that type I collagen-immobilized PCL fibrous scaffolds provided the most significant improvement in the ability to support the attachment and proliferation of all invested cells.

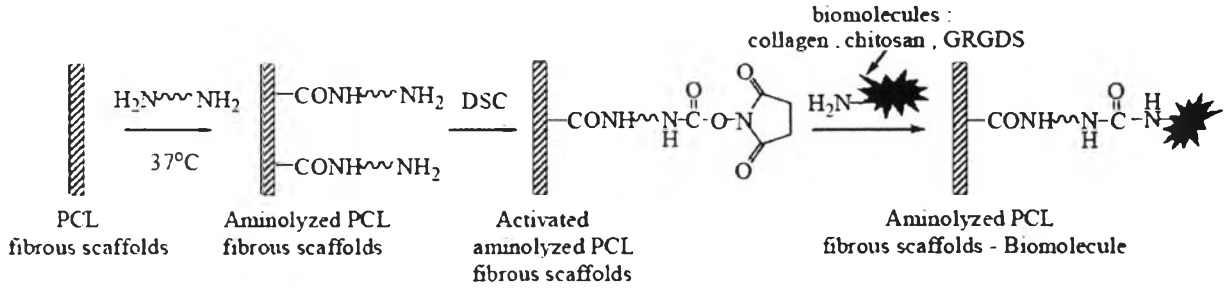


Figure 2.9 Chemical pathway for the immobilization of different biomolecules (i.e., collagen, chitosan, and GRGDS peptide) on the surface of the electrospun PCL fibrous scaffolds. (Mattanavee *et al.*, 2009)

Yang *et al.*, (2002) compared the biocompatibility of PHB, PHBHHx their blends and PLA before and after the surface treatment with lipase and NaOH. The results concluded that cell grew better in the presence of PHBHHx in the blends. For PHB, after treated with either lipase or NaOH, the viable cell number on PHB film increased compared to the untreated PHB. Lipase treatment and NaOH treatment also reduced the pore size on the surface of PHB films. They discussed that both lipase treatment and NaOH treatment improved the hydrophilicity of the films allowed cells easily attached on the polymer films compared to untreated ones. The results were explained that lipase is an enzyme acting to break ester bonds, and produced many hydroxyl group, thus hydrophilicity was improved. So do the NaOH treatment, but NaOH is an strong alkaline which may destroy the polymer structure and reduce the ability of the cells to attach to the treated films.

Yang *et al.*, (2010) treated chitosan on PLA surface through alkaline surface hydrolysis for generating acid groups. Yang et al hydrolyzed PLA surface by treating with 0.25M NaOH/ethanol. Rat osteoblast attachment and proliferation were significantly improved as a result of this treatment.