CHAPTER II
THEORETICAL BACKGROUND AND LITERATURE REVIEW

2.1 Bone

Bone (Figure 2.1) is a specialized type of connective tissue composed of calcified extracellular matrix and three cell types: osteocytes, which are found in cavities within the matrix; osteoblasts, which synthesize the organic components of the matrix; and osteoclasts, which are multinucleated giants cells involved in the resorption and remodeling of bone tissue (Junqueira *et al.*, 2003). Bone is the main constituent of the adult skeleton. Bone functions to protect vital organs, supports fleshy structures, and serves as a reservoir of calcium, phosphate, and other ions.

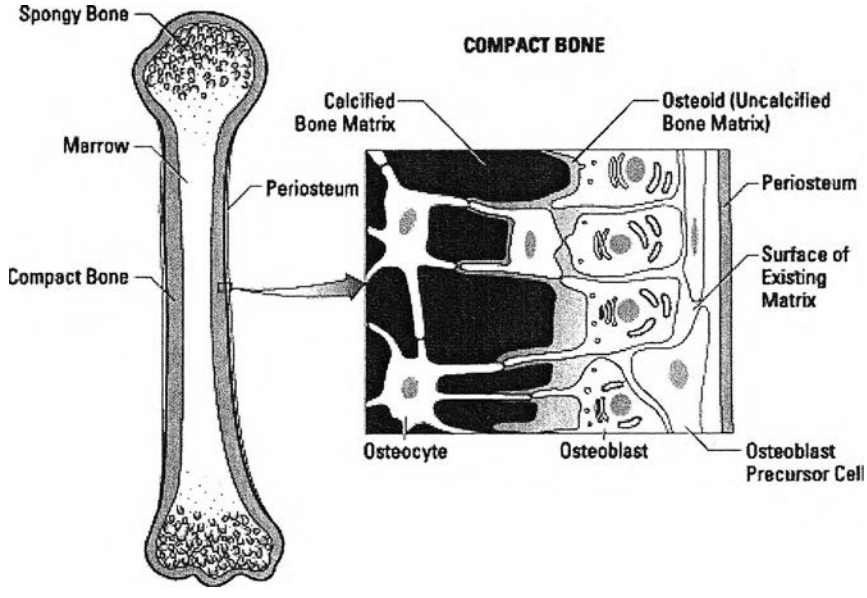

Figure 2.1 Bone matrix.

2.1.1 Bone Matrix

Bone matrix can be classified into two types; organic and inorganic (calcified) matrix. Bone is composed of 33% of organic and 67% of inorganic matrix.

2.1.1.1 Inorganic Portion of the Bone Matrix

Inorganic matter represents about 50% of the dry weight of bone matrix (Gartner *et al.*, 1993). Calcium and phosphorus are especially abundant, but bicarbonate, citrate, magnesium, potassium, and sodium are also found. X-ray diffraction studies have shown that calcium and phosphorus form hydroxyapatite (HAp) crystals with the composition: $Ca_{10}(PO_4)_6(OH)_2$ (Junqueira *et al.*, 2003). However, significant quantities of amorphous (noncrystalline) calcium phosphate are also presented. In electron micrographs, HAp crystals of bone appear as plates that lie alongside the collagen fibrils but are surrounded by ground substance (Junqueira *et al.*, 2003). The surface of HAp is hydrated in which the hydration shell provides the exchange of ions between the crystal and the body fluids.

2.1.1.2 Organic Portion of the Bone Matrix

The organic matter in bone matrix consists of about 90% type-I collagen whereas the remaining 10% is several noncollagenous proteins (Garant *et al.*, 2003). The organic matrix has ground substance which contains proteoglycan aggregates and several specific structural glycoproteins. Bone glycoproteins may be responsible for promoting calcification of bone matrix (Junqueira *et al.*, 2003). The association of minerals with collagen fibers is responsible for the hardness and resistance of bone tissue.

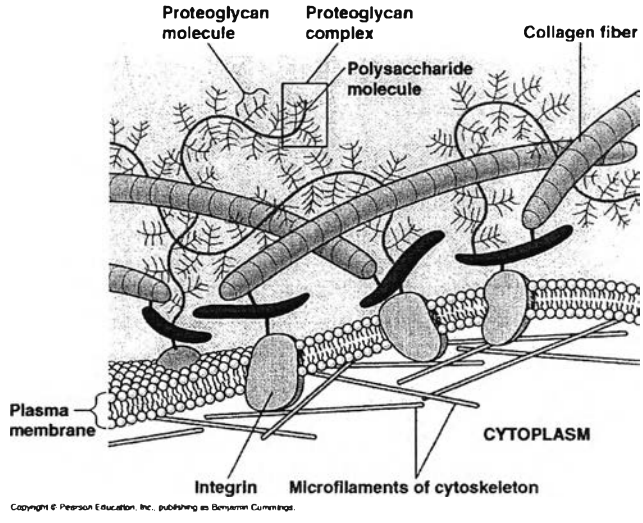

Figure 2.2 The extracellular matrix (ECM)

2.1.2 Organic Matters of Bone

2.1.2.1 Collagen

Collagen is the principal structural protein in the vertebrate body. Collagen is a natural ECM component of many tissues, such as skin, bone, tendon, ligament, and other connective tissues. The extracellular proteins of the main connective tissues consist of 90% or more collagen in tendon and bone, and more than 50% in the skin. Collagen is also found in invertebrates in the body walls and cuticles. It is an important natural material and has been extensively studied in manufactured materials. The term 'collagen' usually implies the collagen present in skin, tendon and bone (Meena *et al.*, 1999). Collagen is the protein unit that polymerizes to form collagen fibrils is the elongated molecule called tropocollagen (Figure 2.3), with 280 nm in length and 1.5 nm in width. Tropocollagen consists of 3 subunit polypeptide chains intertwined in a triple helix (Junqueira *et al.*, 2003). Each molecule of tropocollagen is composed of two α1 and one α2 peptide chains, each with a molecular mass of about 100 kDa, intertwined in a right-handed helix and held together by hydrogen bonds and hydrophobic interactions. Each complete turn of the helix spans a distance of 8.6 nm (Junqueira *et al.*, 2003). Collagen fibrils are thin, elongated structures that have a variable diameter (ranging from 20 to 90 nm) and can be several micrometers in length. They have transverse striation with a periodicity of dark and light bands of 64 nm when the fibrils are observed in the electron microscope (Junqueira *et al.*, 2003). The cross striations are determined by the overlapping arrangement of the tropocollagen molecules. These fibrils associate to form fibers and the fibers can associate to form the bundles. At present, ten different vertebrate collagens have been identified. All collagens contain a unique triple helix. However, the length of the helix and the nature and size of none-helical portions of the molecule vary from type to type. The dominant collagen of skin, tendon and bone is type-I collagen. Type-II collagen is essentially unique to cartilage, and type-III collagen occurs in adult skin (5-10%) in association with type-I and may be minor contaminant of type-I collagen prepared from this source. The other types occur in small amounts and are usually associated with specific biological structures. The safety of collagen for human use is evidenced by its diverse general and biomedical applications. The attractiveness of collagen as a biomedical rest largely on fact that it is a

natural material of low immunogenicity and is therefore considered as a normal constituent rather than as foreign matter by the body. Immunogenicity relates to the capacity of the material to elicit the production of antibodies in animals or humans. Nearly all studies on collagen have shown that it has very low or no immunogenicity. Of the 10 collagen types that have been characterized, type I, III and V are the most desirable for biomedical applications because of high biocompatibility and low immunogenicity (Meena *et al.*, 1999). The fibrillar structure of type I collagen has long been known to be important for cell attachment, proliferation, and differentiated function in tissue culture. In native ECM, collagen exists in a three-dimensional network structure composed of multi-fibrils in the nanofiber scale (50–500 nm).

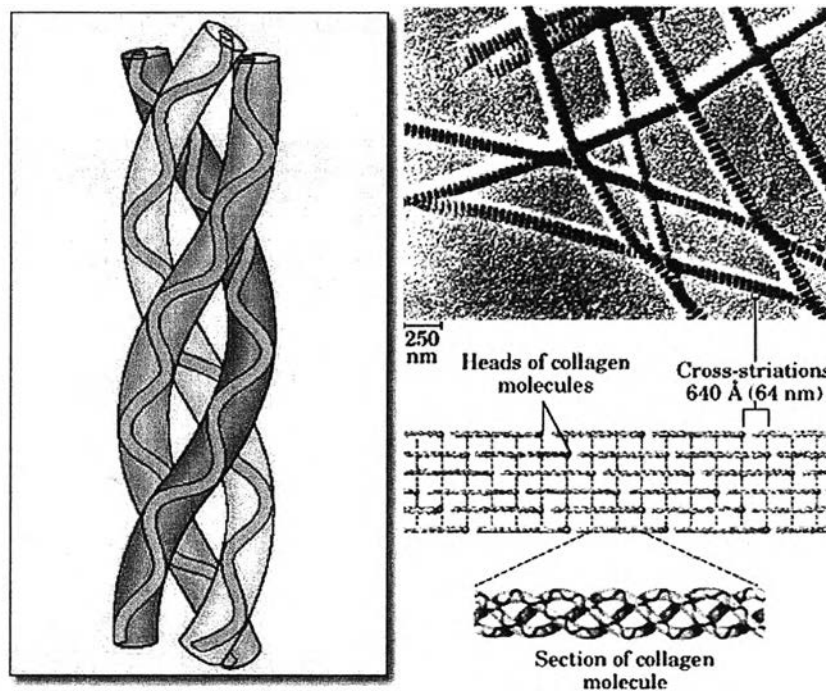

Figure 2.3 Fibrillar structure of collagen molecule.

2.1.2.2 Noncollagenous Proteins

a) Alkaline phosphatase (ALP): ALP is a large group of proteins which is attached to the extracellular surface of cell membranes via a carboxy-terminal glycan-phosphatidyl-inositol (GPI) anchor (Bilezikien *et al.*, 2002). ALP has been recognized as an enzyme particularly associated with sites of rapid calcifi-

cation. ALP functions to catalyze phosphate ions from proteins in alkaline pH in order to prepare phosphate ions for the formation of calcium phosphate matrix and to remove pyrophosphate which is found in serum. The removal of pyrophosphate is beneficial to the calcification of bone matrix due to pyrophosphate can attach on hydroxyapatite crystal which will be obstacle for growing of crystal.

b) Osteonectin: Osteonectin (also called secreted protein, acidic and rich in cystein; SPARC) is the most abundant noncollagenous protein in bone. It is expressed by osteoprogenitor cells, osteoblasts, and newly formed osteocytes. Osteonectin is a 32 kDa protein with calcium- and collagen-binding domains. Although osteonectin has been proposed to have a role in the initiation of mineralization of bone matrix, its exact function is still unclear. According to its ability to bind various collagens and substrate adhesion molecules, osteonectin may have a function in a calcium-mediated organization of extracellular matrices (Garant *et al.*, 2003).

c) Osteopontin: Osteopontin is a charged protein, similar to bone sialoprotein that is expressed in differentiating bone cells. Osteopontin contains several serine phosphorylation sites and a stretch of nine negatively charged aspartic acid residues that bind calcium. Osteopontin also has the RGD tripeptide sequence with specificity toward cell surface integrins (the vitronectin receptor, $\alpha v\beta 3$) (Garant *et al.*, 2003). Osteopontin is concentrated in small globular deposits in bone matrix and in the lamina limitans at the bone surface, in which it plays a role in bone mineralization and in the attachment of osteoblasts and osteoclasts to bone matrix. The concentration of osteopontin in the cement lines that lie between old and new bone segments indicates that it acts as a biologic matrix-bonding agent. Osteopontin is expressed by a variety of cell types and is found in many soft tissues, suggesting that it may have a role in soft tissue organization (Garant *et al.*, 2003).

d) Bone Sialoprotein: Bone sialoprotein, which has a molecular mass of about 33 kDa, contains the RGD tripeptide sequence, a motif interact with the cell surface integrins. Bone sialoprotein contains a stretch of ten glutamic acid residues, providing a negatively charged domain with high calcium-binding potential suggesting that bone sialoprotein can bind tightly to HAp (Garant *et al.*, 2003). Calcium-binding proteins, such as bone sialoprotein and osteopontin, have been shown to inhibit mineral deposition when present in solution. However, when bound

to a solid substrate they can promote mineral deposition. It has been proposed that the association of osteocalcin and/or bone sialoprotein with collagen fibrils creates locally high concentrations of calcium, leading to precipitation of mineral (Garant *et al.*, 2003). Bone sialoprotein also increases osteoclastic resorption by promoting greater adhesion of osteoclasts to bone matrix molecules.

f) Osteocalcin: Osteocalcin is a low-molecular weight protein containing three α-carboxyglutamic acid residues per molecule (also called GLA protein). Osteocalcin is one of the most abundant noncollagenous proteins of bone matrix. Vitamin K is required for the synthesis of the α-carboxyglutamic acid residues. These residues provide calcium-binding sites that are believed to play a role in bone matrix mineralization or in the regulation of crystal growth (Garant *et al.*, 2003). The function of osteocalcin in bone mineralization is supported by the observation that osteocalcin messenger ribonucleic acid (mRNA) is localized in osteoblasts and simultaneously in the mineralized bone matrix. Osteocalcin has since been localized over the mineralized portion of bone and in acellular cementum (Garant *et al.*, 2003).

2.1.3 Bone Cells

Bone cells are classified into four types as following.

2.1.3.1 Osteoprogenitor Cells

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

2.1.3.2 Osteoblasts

Osteoblasts are derived from osteoprogenitor cells which are responsible for the synthesis of the organic components of bone matrix (i.e. type I collagen, proteoglycan, and glycoproteins). Osteoblasts are located at the surface of bone tissue. During matrix synthesis, osteoblasts have a cuboidal to columnar shape, basophilic cytoplasm, and well-developed rough endoplasmic reticulum (rER) and Golgi complex. When their synthesizing activity declines, they become flatten and cytoplasmic basophilia declines (Junqueira *et al.*, 2003). Matrix components are secreted at the cell surface, which is in contact with older bone matrix, producing a layer of new matrix, called osteoid (uncalcified bone matrix), between the osteoblasts

layer and the previously formed bone. This process, bone apposition, is completed by subsequent deposition of 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.

2.1.3.3 Osteocytes

Osteocytes, which derived from osteoblasts, are mature bone cells housed in the lacunae. Only one osteocyte is 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).

2.1.3.4 Osteoclasts

Osteoclasts are very large, motile, and multinucleated cells (up to 50 nuclei) (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 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).

2.1.4 Some Types of Osteoblast-like Cells

2.1.5.1 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).

2.1.5.2 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).

2.1.5.3 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).

2.1.5 Calcification of Bone

Mechanism of calcification of bone is still unclear, although it is known to be stimulated by certain proteoglycans and the Ca^{2+}-binding glycoprotein, osteonectin and bone sialoprotein. One theory, called heterogeneous nucleation, is that calcification begins when there are deposits of calcium phosphate on the collagen fibrils. Crystallization occurs in the gap region of the collagen fibers. Once this region has nucleation, calcification proceeds.

Another theory, the most commonly accepted theory, of calcification is based on the presence of matrix vesicles within the osteoid. Osteoblasts release matrix vesicles which contain a high concentration of Ca^{2+} and PO_4^{3-} ions, cyclic adenosine monophosphate (cAMP), adenosine triphosphate (ATP), adenosine tri-

phosphatase (ATPase), ALP, pyrophosphate, calcium-binding proteins, and phosphoserine (Gartner *et al.*, 2001). The matrix vesicle membrane posses numerous calcium pumps, which transport Ca^{2+} ions into the vesicle. When the concentration of Ca^{2+} ions within the vesicle increases, crystallization occurs and growing HAp crystal pierces the membrane, bursting the matrix vesicle and releasing its contents. The HAp crystals released from the matrix vesicles act as nidi of crystallization (Gartner *et al.*, 2001). The high concentration of ions in their vicinity, along with the calcification factors and calcium-binding proteins, support the calcification of the matrix.

2.1.6 Process of Bone Remodeling

The cycle (Figure 2.4) where new bone formation and resorption occur, is referred to as bone remodeling. Osteoclasts (bone-resorbing cells) are transported to the bone by blood, and attach themselves to the bone and release acids and enzymes, which resorb the existing bone structure. After the osteoclasts resorb the bone and disappear, osteoblasts appear 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 forms.

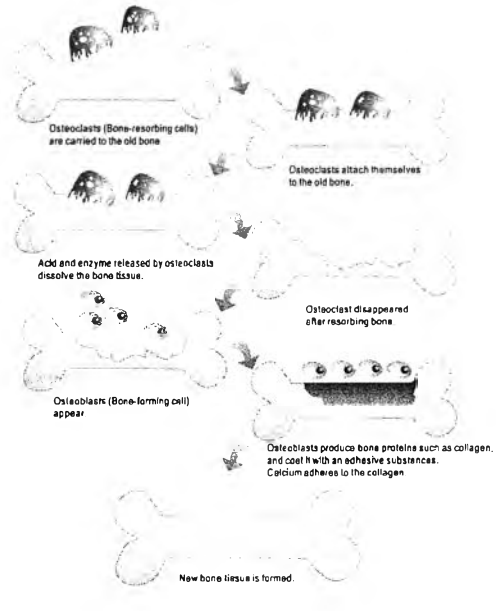

Figure 2.4 Bone remodeling cycle.

2.2 Tissue Engineering

Recently, organ and tissue loss or failures resulting from an injury or other types of damage become a major human health problem. Tissue or organ transplantation is a standard therapy to treat these patients, but this is severely limited by donor shortage. Other available therapies including surgical reconstruction, drug therapy, synthetic prostheses, and medical devices are not limited by supply, but they do have other problems such as infection, pathogens, immune rejection, etc.

Tissue engineering has emerged as a promising alternative approach to treat the loss or malfunction of a tissue or organ without the limitations of current therapies. Tissue engineering is an innovative technology that combines materials engineering, cellular biology, and genetic engineering. It involves the expansion of cells from a small biopsy, followed by the culturing of the cells and signaling molecules (i.e. growth factors) in temporary three-dimensional scaffolds. After that the scaffolds were implanted into the defect to induce and direct the growth of normal, healthy bone as the scaffolds degrade (Figure 2.5). By using the patient's own cells, this approach has the advantages of autografts, but without the problems associated with adequate supply. It is then logical to say that an appropriate 3D scaffold is an essential component for a tissue engineering strategy.

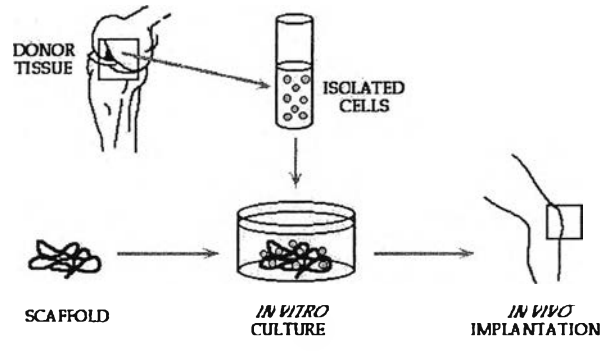

Figure 2.5 Scaffold-guided tissue regeneration.

Ideally, scaffolds for tissue engineering should meet several design criteria: (i) the surface should permit cell adhesion, promote cell growth, and allow the retention of differentiated cell functions; (ii) the scaffolds should be biocompatible, neither the polymer nor its degradation by-products should provoke inflammation or toxicity in vivo; (iii) the scaffold should be biodegradable and eventually eliminated; (iv) the porosity should be high enough to provide sufficient space for cell adhesion, extracellular matrix regeneration, and minimal diffusional constraints during culture, and the pore structure should allow even spatial cell distribution throughout the scaffold to facilitate homogeneous tissue formation; (v) the material should be reproducibly processable into three-dimensional structure, and mechanically strong. A number of three-dimensional porous scaffolds fabricated from various kinds of biodegradable materials have been developed and used for tissue engineering of liver, bladder, nerve, skin, cartilage, ligament, bone, etc. (Chen *et al.*, 2002; Salgado *et al.*, 2004)

2.3 Biomaterials Applied for Tissue Engineering

Up to now several materials such as metals, ceramics and polymers from both natural and synthetic origins have been proposed. However, metals and most of the ceramics are not biodegradable, which leaves the researcher's choice reduced to a small number of ceramics and to biodegradable polymers.

Biodegradable polyesters such as poly(lactide-*co*-glycotide) (PLGA), polycaprolactone (PCL), poly(lactic acid) (PLA), and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) have been attractive biomaterials being used as scaffolds in tissue engineering because they degrade to natural metabolites and may be tailored to particular applications by manipulation of shape, porosity, and degradation rate, and so forth (Hsu *et al*, 2004; Wutticharoenmongkol *et al.*, 2007; Chen, *et al.*, 2007; Sombatmankhong *et al.*, 2006).

2.3.1 Poly(lactic acid) (PLA)

Poly(lactic acid) (PLA) (Figure 2.6) is a thermoplastic polyester synthesized from ring opening polymerization of lactides. Lactides are cyclic diesters of

lactic acid. Due to the chiral nature of lactic acid, three forms of polylactide exist: poly-L-lactide (PLLA), poly-D-lactide (PDLA), and poly-DL-lactide (PDLLA). It is currently being investigated as a material for regeneration of several tissues including cartilage, bone, liver, and intestine. PLA is widely used because of its robust mechanical properties and the ability to be processed into scaffolds of different shapes. PLA has a crystallinity around 37%, the glass transition temperature, T_g around 60–70°C is followed by melting behavior between 150 and170°C (Gupta *et al.*, 2007). The presence of ester linkages in the polymer backbone allows gradual hydrolytic degradation of the polymer. The degradation products, carbon dioxide and water, are endogenous compounds and as such are non-toxic. In addition, PLA degradation rate is matched with the healing time of damaged human tissues. PLA has therefore gained the approval of US Food and Drug Administration (FDA) for a variety of human clinical applications (Cui *et al.*, 2003).

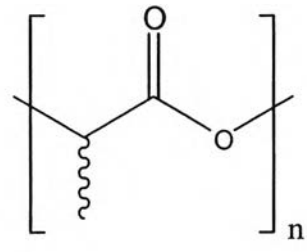

Figure 2.6 Chemical structure of poly(lactic acid) (PLA).

2.3.2 Biodegradation of Polymer

The main factors that determine the rate of degradation are:

2.3.2.1 Chemical Stability of the Polymer Backbone

The rate of degradation depends on chemical structure of polymer. For example, anhydride bonds tend to be hydrolyzed faster than ester bonds (Ratner *et al.*, 1996).

2.3.2.2 Hydrophobicity

The rate of degradation depends on the rate or ability of water penetration into polymer. The hydrophobic polymer shows slower rate of degrada-

tion than that of the hydrophilic polymer. For example, PCL degrades slower than PLA (Pitt *et al.*, 1981) and PLA degrades slower than PGA (Ratner *et al.*, 1996).

2.2.2.3 Morphology of the Polymer

Polymer with high crystallinity offers the slow rate of hydrolysis reaction since the densely packed of crystalline is the resistance for penetration of water into matrix. For example, PLLA, which is semicrystalline polymer, tends to degrade slower than PDLLA, which is amorphous polymer (Ratner *et al.*, 1996). *2.2.2.4 The Fabrication Process*

Polymer in the form of highly porous microspheres degrades faster than the same polymer which is produced in the form of dense microspheres (Ratner *et al.*, 1996).

2.3 Electrospinning

The electrospinning process (Figure 2.7) is an economical and simple approach which involves the application of a strong electrical potential to a polymeric liquid contained in a reservoir (e.g., syringe) attached to a metal nozzle (e.g., needle) across a finite distance between the nozzle and a grounded collector (i.e., collection distance). Upon increasing the applied electrostatic field strength (i.e., electrical potential divided by collection distance) to a critical value, a pendant droplet of the polymeric liquid at the tip of the nozzle gradually changes its shape from partially-spherical into conical. Further increase in the electrostatic field strength causes an ejection of a stream of charged liquid (i.e., charged jet) from the apex of the liquid cone. The jet accelerates towards and finally rests on the grounded collector. Owing to the high enough viscosity of the polymeric liquid, the ejected, charged jet remains stable and does not break up into spherical droplets as commonly found in the electrospraying of low molecular weight liquids. This results in the deposition of ultrafine polymeric fibers with extremely long length and high specific surface area on the collector as a non-woven mat. With these special fine properties, electrospinning has been employed as a new approach for preparing suitable fibrous structures for tissue engineering applications (Wutticharoenmongkol *et al.*, 2007; Chen *et al.*, 2007). Electrospun scaffolds have fibrous structures, which are similar to that of the

natural extracellular matrix (ECM). In addition, fibrous scaffolds fabricated by electrospinning have many other advantages, including the high porosity, interconnected pores and relatively large surface areas. All of these structural characteristics promote favorable responses of seeded cells in vitro, such as enhanced cell attachment and proliferation (Yoshimoto *et al.*, 2003; Min *et al.*, 2004; Venugopal *et al.*, 2008).

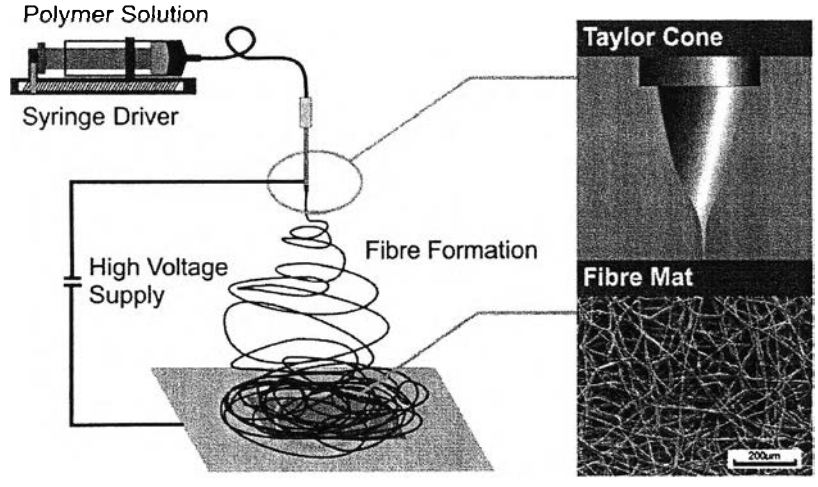

Figure 2.7 Schematic diagram of electrospinning system.

2.4 Surface Modification

Polyesters are hydrophobic due to non-polar groups along its backbone, which results in a scaffold difficult to infiltrate with culture media. It is well known that substrate attachment sites are necessary for growth, differentiation, replication and metabolic activity of most cell types in culture. The initial attachment of a cell to a substrate is mediated by cell-surface adhesion factors. Intensive research has shown that cell-extracellular matrix (ECM) interactions participate directly in promoting cell adhesion, migration, growth and differentiation. Various ECM molecules such as type-I collagen, fibrinogen and fibronectin contain specific amino acid receptors that allow them to bind directly to cell-surface receptors. Other studies have also demonstrated the binding functions of the arginine–glycine–aspartic (RGD) peptide sequence in some extracellular matrix molecules. Based on these fundamental findings,

studies have been conducted to mimic the extracellular matrix on substrate surfaces, by immobilizing extracellular molecules or RGD peptides to the scaffold surface (Hsu *et al.*, 2006). There are several methods of immobilizing proteins on a substrate for biomedical and biochemical applications. These can be divided into two general categories: physical and chemical. Various workers have demonstrated that coating the scaffold surface with collagen, or pre-soaking scaffolds in media improves cell seeding efficiency and spreading (Yang *et al.*, 2003). Physical coating is a simple technique; however, this coating is less biologically stable.

Four major protein immobilization techniques are currently used. These are: (i) covalent binding, in which attachment of the active component to the substrate surface uses a chemical reaction or linkage to activated surface groups (Wang *et al.*, 2003; Yang *et al.*, 2003); (ii) entrapment, which is a physical trapping 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 (Karakeçili *et al.*, 2008); and (iv) adsorption, which applies a protein solution to a substrate or film and allows the molecule to adsorb to the substrate over a specified time period (Woo *et al.*, 2007).

Zhu *et al.* (2004b) developed several methods to enhance the cell-polymer interactions. Poly-L-lactic acid (PLLA) was selected as matrix being modified by 1) aminolysis (PLLA-NH_2), 2) collagen immobilization with glutaraldehyde (PLLA-GA-Col), 3) chondroitin sulfate (CS)/collagen layer-by-layer (LBL) assembly (PLLACS/Col), 4) photo-induced grafting copolymerization of hydrophilic methacrylic acid (MAA) (PLLA-g-PMAA), and 5) further immobilization of collagen with 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDAC) (PLLA-g-PMAA-Col). In general, all the modifications can apparently improve cell response to the substrate PLLA, resulting in an improved cytocompatibility. All the collagen-modified PLLA showed more positive cell response than those purely aminolyzed or PMAA grafted. Among all the methods, collagen immobilization by LBL assembly or GA bridging after aminolysis are more acceptable for the convenience and applicability to scaffolds. These two methods are suitable for both plane membrane and

scaffolds with irregular shape and inner structure, where traditional methods are generally unavailable. These methods are not restricted to collagen and can be extended to other biomacromolecules such as cell growth factors, cell morphology generation proteins, polysaccharides, and so forth.

2.4.1 Immobilization of Biomolecules onto Polyester Surface

Due to the lack of chemical functionalities, it is usually difficult for polyesters to favor polymer–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 introduced on the surface of polymeric scaffolds.

Zhu *et al.* (2004c, 2006) showed that the aminolysis reaction successfully generated free amino groups on the poly(L-lactic acid) (PLLA) and poly(L-lactic-co-caprolactone) (PLLC) membrane surface by reaction with 1,6-hexanediamine. Then biomacromolecules such as gelatin, chitosan, collagen, fibronectin were further covalently immobilized as well by employing glutaraldehyde as a coupling agent. Alternative coupling agent such as *N, N'*-disuccinimidyl carbonate (DSC) can be used (Mattanavee *et al.*) as well. Aminolyzed polycaprolactone (PCL) film or electrospun fiber mat were treated by DSC, the new linkages —succinimidyl esters— were generated which is less prone to hydrolysis. After that, biomolecules were further immobilized in the water (Figure 2.8).

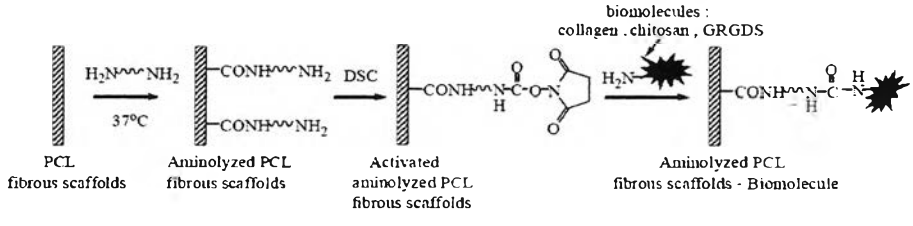

Figure 2.8 Chemical pathway for the immobilization of different biomolecules (i.e., collagen, chitosan, and GRGDS peptide) on the surface of the electrospun PCL fibrous scaffolds (Mattanavee *et al.*)

The existence of NH_2 groups on polyester surface was characterized by ninhydrin or fluorescamine analysis. Figure 2.9 shows that NH_2 density increased with 1,6-hexanediamine concentration. However, when the concentration of 1,6-hexanediamine is equal to or greater than 0.1 g/ml, the mechanical properties of the PLLA membrane became worse. A similar tendency relative to NH_2 amount was found in Figure 2.10: again, when the aminolyzing time is equal to or longer than 20 min, the PLLA membrane was also greatly degraded. To maintain enough mechanical properties for practical application, the selected condition was aminolyzed at 50°C for 8 min in a 1,6-hexanediamine–propanol solution (0.06 g/m). The NH_2 density on PLLA membrane aminolyzed under selected conditions was about 1.6×10^{-7} mol/cm^2. The similar trend was found for aminolyzed PLLC; however, there were some saturation effect. Figure 2.11 shows that NH_2 density increased with 1,6-hexanediamine concentration and saturated at high concentration. The similar effect was found in the reaction-time dependence. Scanning force microscopy, SFM measurement was used to observe surface topology. Figure 2.12 shows that after aminolysis, the PLLA membrane surface became rougher. The surface alteration after the PLLC membrane was aminolyzed and protein immobilized was also studied by XPS (Table 2.1). Increasing in %N which presented on the surface confirmed the existence of the amino group and biomolecules on PLLC membrane surface. Water contact angle measurements evaluated the wettability of modified and unmodified PLLC surfaces. Table 2.2 shows that hydrophilicity was increased after aminolysis (83.4° to 78.5°) and then it was obviously increased after the biomolecules was bonded. The culture of human umbilical vein endothelial cells (HUVECs) *in vitro* proved that the cell proliferation rate and cell activity of both aminolyzed and biomacromolecule-immobilized PLLAs were improved compared with control PLLA. The similar result was seen with modified PLLC. PLLC grafted with either fibronectin or collagen supported the attachment, growth and functional morphology of all three types of cells important in natural esophagus. Mattanavee *et al.* found that among the various biomolecule-immobilized PCL fibrous scaffolds, the ones that had been immobilized with type-I collagen, a RGD-containing protein, showed the greatest ability in supporting both the attachment and proliferation of all the cell types investigated.

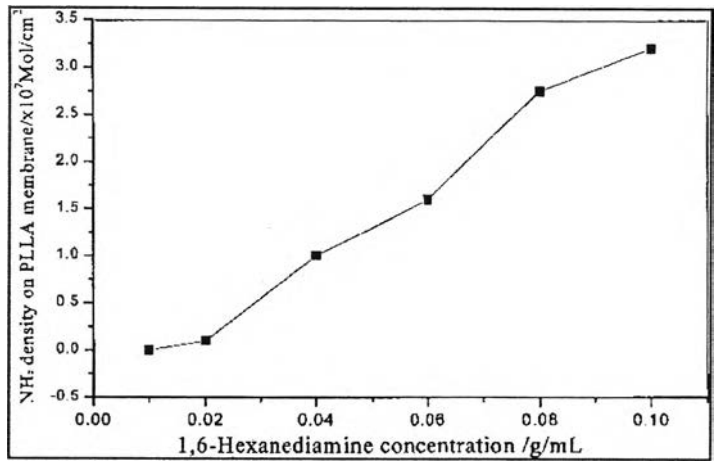

Figure 2.9 NH_2 density on PLLA surface as a function of the concentration of 1,6-hexanediamine–propanol solution. The PLLA membrane was aminolyzed at 50°C for 8 min (Zhu *et al.*, 2004c).

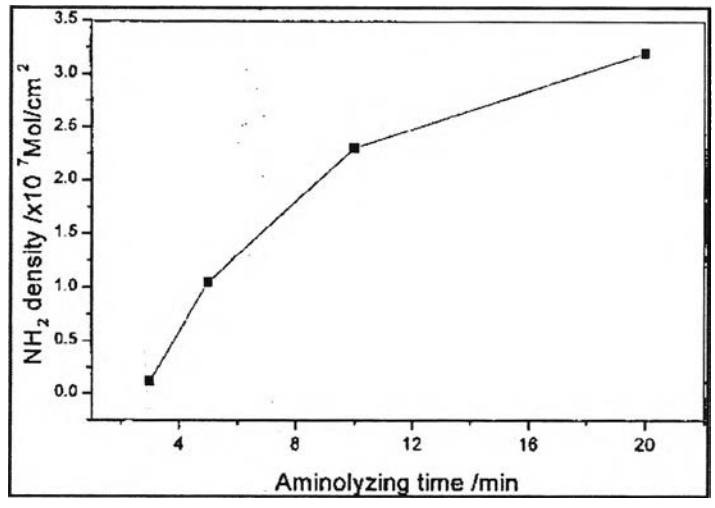

Figure 2.10 NH_2 density on PLLA surface as a function of aminolyzing time. The aminolysis reaction of PLLA membrane took place at 50°C in 1,6-hexanediamine–propanol solution (0.06 g/ml) (Zhu *et al.*, 2004c).

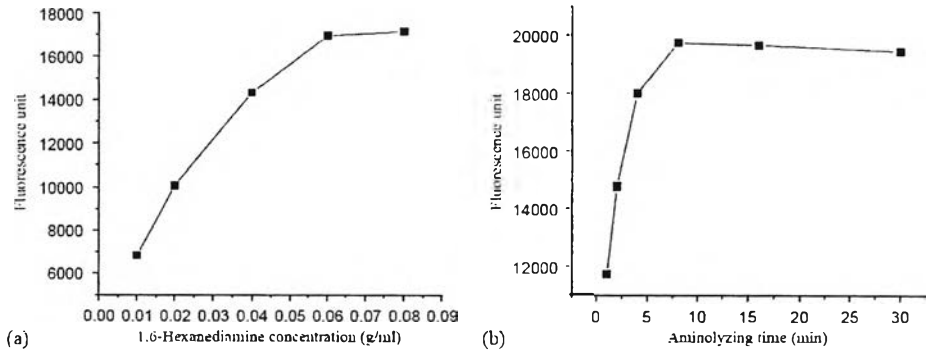

Figure 2.11 The fluorescence intensity as a function of (a) 1,6-hexanediamine concentration (for a 2 min reaction) and (b) aminolyzing time (with 0.06 g/ ml 1,6-hexanediamine solution) at 21°C for PLLC membrane (Zhu *et al.*, 2006).

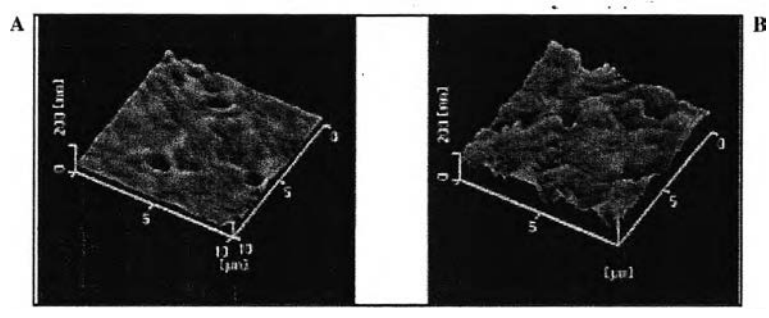

Figure 2.12 Surface morphology of control PLLA membrane (A) and PLLA membrane aminolyzed at 50°C for 8 min in 1,6-hexanediamine–propanol solution (0.06 g/ml) (B) (Zhu *et al.*, 2004c).

Table 2.1 Atomic concentration (%) of C_{1s}, N_{1s}, O_{1s} and Si_{2p} for modified and unmodified PLLC membranes. The atomic concentration of fibronectin and collagen were herein used as reference (Zhu *et al.*, 2006)

Sample	C_{1s}	N_{1s}	O_{1s}	Si_{2p}	O_{1s}/C_{1s}
PLLC	65.57	0	33.32	1.11	0.50
PLLC-NH_2	66.97	0.72	31.42	0.88	0.46
PLLC-Fn	66.78	6.45	25.53	1.24	0.38
PLLC-Col	67.14	1.56	30.30	1.01	0.45
Fn	63.38	13.61	22.97	0	0.36
Col	67.38	12.73	19.89	0	0.30

Table 2.2 Water contact angles of the control and modified PLLC membranes (Zhu *et al.*, 2006)

Samples	Water contact angle/degree (Sessile drop)
Control PLLC	83.4 ± 1.1
PLLC-NH_2[a]	78.5 ± 1.4
PLLC-Fn[a]	66.5 ± 2.3
PLLC-Col[a]	69.2 ± 1.3
PLLC-ad-Fn[b]	68.4 ± 6.9
PLLC-ad-Col[b]	71.8 ± 3.6

[a]The PLLC membrane was prepared in 0.06 g/ml 1,6-hexanediamine solution at 21 °C for 2 min.
[b]The PLLC membrane was immersed in 1 mg/ml fibronectin and collagen solutions for 24 h, respectively, followed by the rinsing process same as PLLC-Fn and PLLC-Col.

The present contribution focuses on improving surface hydrophilicity of electrospun (e-spun) PLA fiber mats by aminolysis with 1,6-hexamethylenediamine to introduce free amino groups onto a ester-containing polymer surface. Type-I collagen was subsequently immobilized with *N, N'*-disuccinimidyl carbonate (DSC) as a coupling agent. Indirect cytotoxicity evaluation of the aminolyzed, activated, collagen-immobilized PLA e-spun fiber membranes based on mouse fibroblasts (L929) and pre-osteoblasts (MC3T3-E1) was investigated. Morphological appearance of pre-osteoblasts (MC3T3-E1) cultured on these modified e-spun fiber mats was observed. The potential use of the e-spun fiber mats as bone scaffolds was further evaluated *in vitro* with MC3T3-E1 in terms of attachment, proliferation, and differentiation of the cells that were cultured on the scaffolds. For differentiation study, the ALP activity was used as an early marker of differentiation. The results were compared with those of TCPS.