CHAPTER II
LITERATURE REVIEW

In this chapter, theoretical background and literature review of bone, polycaprolactone, electrospinning process, and bone tissue engineering are concerned.

2.1 Bone

Bone 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.

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.

2.1.2 Organic Matters of Bone

2.1.2.1 Collagen

The protein unit that polymerizes to form collagen fibrils is the elongated molecule called tropocollagen, with 280 nm in length and 1.5 nm in width (Junqueira *et al.*, 2003). Tropocollagen consists of 3 subunit polypeptide chains intertwined in a triple helix. For type I collagen, 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.

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 calcification. 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 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 triphosphatase (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.

2.1.5 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).

2.2 Polycaprolactone (PCL), Poly(lactic acid) (PLA), and Poly(glycolic acid) (PGA)

2.2.1 General Properties of PCL, PLA, and PGA

Polycaprolactone (PCL), poly(lactic acid) (PLA), and poly(glycolic acid) (PGA) are biodegradable, thermoplastic, aliphatic polyester. Their chemical structures are shown in Figure 2.1

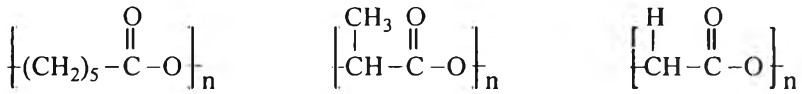

Figure 2.1 The chemical structures of polycaprolactone (PCL), poly(lactic acid) (PLA), and poly(glycolic acid) (PGA).

PCL is synthesized from ring opening polymerization of ε-caprolactone rings as shown in Figure 2.2.

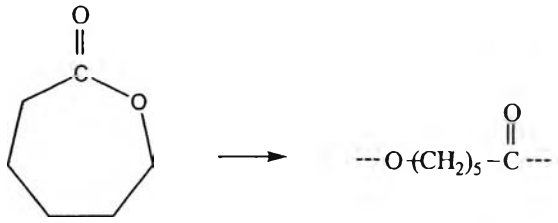

Figure 2.2 The reaction of ring opening polymerization of ε-caprolactone rings.

PCL is a semicrystalline polymer with a melting point near 62°C and a glass transition temperature about -60°C (Harper *et al.*, 2002).

PLA 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). PLLA has a crystallinity around 37%, a glass transition temperature between 50-80° C and a melting temperature between 173-178° C (wikipedia, 2007). The polymerization of a racemic mixture L- and D-lactides leads to the synthesis of poly-DL-lactide (PDLLA) which is not crystalline but amorphous. Another interesting biodegradable polymer is polyglycolide (PGA). PGA is also a thermoplastic polyester but from glycolic acids. PGA has a glass transition temperature between 35-40°C and its melting point is reported to be in the range of 225-230° C. PGA has crystallinity around 45-55% (wikipedia, 2007).

2.2.2 Biodegradation of Polymer

The main factors that determine the rate of degradation are:

2.2.2.1 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).

2.2.2.2 Hydrophobicity

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

degradation 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).

PCL capsules with initial molecular weight (Mw) of 66000 remained intact in shape during 2 years of implantation. It broke into low molecular weight (Mw = 8000) pieces at the end of 30 months. The molecular weight (Mw) of PCL decreased with time and followed a linear relationship between log Mw and time (Sun *et al.*, 2006).

Pitt *et al.* (1981) reported the mechanism of *in vivo* degradation of some aliphatic polyesters; PDLLA, PCL, and their copolymer in rabbit. The proposed mechanism of degradation of these polyesters involves two stages. The first stage is chain scission by non-enzymatic random hydrolytic ester cleavage which resulting in decreasing of molecular weight and intrinsic viscosity. The rate of chain scission depends on the initial molecular weight, morphological and chemical structures of polyesters. Mass loss is not appeared in this stage. The second stage involves weight loss and higher rate of chain scission, decreasing of molecular weight and intrinsic viscosity. They also reported that the rate of weight loss is enhanced when the polyesters were introduced as powders or if the melting point is below body temperature.

Sun *et al.* (2006) also reported the similar mechanism of degradation that the first stage involves a decrease in molecular weight without mass loss and deformation and the second stage begins when the material break into pieces and

mass loss occurs. They therefore predicted that the materials would then gradually be absorbed and excreted by the body. They also found that in the first stage, *in vivo* degradation rate of PCL in rat is identical to the *in vitro* hydrolysis at 40°C and obeyed first-order kinetics.

For PCL, Pitt *et al.*, (1981) showed that the rate of weight loss was greater and the period prior to weight loss was shorter when the comonomer content was sufficient to reduce the melting point of ε-caproate sequences to body temperature.

Pitt *et al.* (1981) studied the degradation of poly(ε-caprolactone-*co*-DL-lactide) with four different copolymer compositions. All of the copolymers degraded much more rapidly than their component homopolymers, with short induction periods prior to rapid weight loss.

Lu *et al.* (2000) studied *in vitro* degradation of porous poly(DL-lactic-*co*-glycolic acid) (PLGA) foams in pH 7.4 phosphate-buffered saline (PBS) at 37°C and *in vivo* degradation in rat mesentery. PLGA 50:50 exhibited significantly shorter half-lives compared to PLGA 85:15 foams both *in vitro* and *in vivo*. Moreover, PLGA 50:50 exhibited significantly faster degradation *in vivo* as compared to *in vitro* conditions due to an autocatalytic effect of the accumulated acidic degradation products in the medium surrounding the implants.

The autocatalytic effect from acidic products is also a factor in degradation process (Michel, 1994; Lu, 2000). Michel *et al.* (1994) reported that the interior of large size devices of PLA/GA degraded faster than the outer zone. Their explanation based on diffusion-reaction phenomena combined with the autocatalytic effect of carboxylic chain ends. Two phenomena are of critical importance. First, degradation causes an increase in the number of carboxylic chain ends which are known to autocatalyse ester hydrolysis. Second, only oligomers which are soluble in the surrounding aqueous medium can escape from the matrix. As degradation time increases, soluble oligomers which are close to the surface can leach out, whereas those located inside the matrix remain entrapped and totally contributed to the autocatalytic effect.

The increasing of crystallinity along with degradation was observed by Ali *et al.* (1993). It seems that degradation is initiated in the amorphous phase and then in the crystalline regions because water molecules diffuse through the amorphous phase more easily than through the crystalline phase. The fragments after degradation become more motile and then recrystallization occurs and crystallinity increases.

The removal or excretion of residues out of body after degradation of PCL is also questionable. There was evidence that the residues could be excreted from body (Sun *et al.*, 2006). The excretion of PCL residues was traced in rats by radioactive labeling. After the particular time of degradation, low molecular weight PCL pieces were found to be metabolized and excreted from the body through urine and feces. The materials did not accumulate in any body organs confirmed by that the radioactivity in the organs was all close to the background level.

2.3 The Electrospinning Process

Electrospinning is a fiber spinning technique that produces polymer fibers of nanometer to micrometer range in diameters. The polymer solutions or polymer melts are placed into a container that has a millimeter size nozzle and are charged to a high electric potential that produces a high electric field between a nozzle and a grounded collecting screen. When the electric field reaches a critical value at which the repulsive electric force overcomes surface tension at the surface of polymer solutions or polymer melts, a charged jet forms. As the jet travels in air, the solvent evaporates, leaving behind a charged polymer fiber which lays itself randomly on a collecting metal screen. Thus, continuous fibers are laid to form a non-woven fabric (Doshi *et al.*, 1995). Figure 2.3 shows a schematic drawing of the electrospinning apparatus.

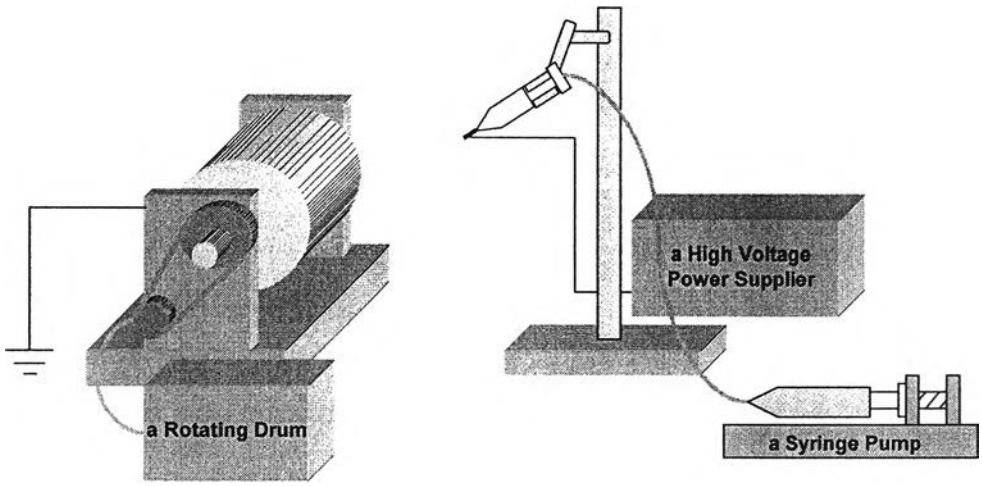

Figure 2.3 A schematic drawing of the electrospinning apparatus.

The formation of fibers from this spinning process can be divided into two parts:

2.3.1 The Initiation of the Jet

Before the electric field is applied to the polymer solutions, and when the capillary tube are in a vertical position and carries a drop at the tip of nozzle, the relation between the surface tension and the height of the column of liquid under equilibrium conditions is given by

$$2\gamma(1/R + 1/r) = \rho gh \qquad (1)$$

where γ is the surface tension of the liquid of density ρ, h is the height of the column of liquid above the lowest surface of the drop, R is the radius of curvature of the liquid at the upper liquid surface and r is the radius of curvature of the liquid at the lower surface of the liquid (Michelson, 1990).

Consider a droplet of polymer solutions that is applied to a high electric field. Charges that flow onto liquid surface repel each other. The repulsion forces are opposed to the forces from surface tension. The polymer droplet becomes unstable when the charges distributed on the surface overcome the surface tension. The conditions that are necessary for a charged surface to become unstable are described by the equilibrium equation as following.

$$V_* = (4\pi r\gamma)^{1/2} \quad (2)$$

V_* is the critical potential, r is the droplet radius, and γ is the surface tension of the solutions (Koombhongse, 2001). For the droplets subjects to a higher potential, $V > V_*$, the droplet elongates into a cone-like shape that was described mathematically by Taylor and often referred to as a Taylor cone (Taylor, 1969).

As the potential is increased and reached the maximum instability of the liquid surface, a jet of liquid is ejected from the tip of the cone. Taylor (1969) showed that the critical voltage V_c (expressed in kilovolts) at which the maximum instability develops is given by

$$V_c^2 = 4H^2/L^2 (\ln 2L/R - 1.5)(0.117\pi R\gamma) \quad (3)$$

where H is the distance between the electrodes, L and R are the length and radius of the capillary, respectively, and γ is the surface tension.

2.3.2 The Continuous Flow of the Jet

The mechanism of the appearance of a stable electrospinning jet is evidently established by the observation of the jet formation through the high speed electronic camera which recorded up to 2000 frames per second with a time resolution of approximately 0.0125 ms (Reneker *et al.*, 2000).

There are two kinds of electrical forces that act on the jet: the external field that tries to pull the jet toward collector and the self-repulsion between the charges carried by adjacent segments of the jet that try to push each other apart. The self-repulsion can also cause different types instability such as bending instability and splitting instability.

In bending instability, or whipping instability, the jet rotates in a conical region, whose vertex is the end of the straight jet. The other end of the jet, which is highly stretched, and reduced in diameter, is deposited on the collector as a result of the fast whipping motions (Shin *et al.*, 2001).

After some time, segment of a loop suddenly developed a new bending instability, but at a smaller scale than the first. Each cycle of bending instability can be described in three steps (Reneker *et al.*, 2000).

Step (1) A smooth segment that was straight or slightly curved suddenly developed an array of bends.

Step (2) The segment of the jet in each bend elongated and the array of bends became a series of spiraling loops with growing diameters.

Step (3) As the perimeter of the loops increased, the cross-sectional diameter of the jet forming the loop grew smaller; the conditions for step (1) were established on a smaller scale, and the next cycle of bending instability began.

The other instability of the charged jet is the splitting instability. It occurs when the charge density of the charged jet increases as the solvent evaporates. The charged jet can reduce its charge per unit surface area by ejecting a smaller jet from the surface of the primary jet, or by splitting apart to form two smaller jets (Koombhongse *et al.*, 2001).

2.4 Tissue Engineering and Electrospun Fibers

2.4.1 Introduction of Tissue Engineering

Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences to the development of biological substitutes that restore, maintain or improve tissue function. Biomaterials play an important role in tissue engineering by serving as matrices for cellular ingrowth, proliferation, and new tissue formation in three-dimensions. Tissue engineering has been recognized as a way to repair or reproduce suffered tissue. In the living system, the extracellular matrix (ECM) plays an important role in mechanical supporting and controlling cell behavior. ECM provides organization of cells in space and signaling cell regulation. ECM is composed of a ground substance (i.e. proteoglycan) and fibrous protein (i.e. collagen, elastin). Collagens embedded as a three-dimensional (3D) fibrous network linking with proteoglycans. Fibrous network of collagen is formed in hierarchically by nanometer scale multi-fibrils where cells are in micrometer range. The goal of the scaffolds design is to produce the structure that

can replace the natural ECM until host cells can regenerate and resynthesize a new natural matrix.

An ideal scaffold possesses five characteristics (Hutmacher, 2001). First, it possesses the appropriate 3D shape and size suitable for repairing at the implant site. Second, it possesses mechanical properties similar to those of the tissue in which it is implanted so that it can support local stresses until new tissue develops. Third, the surface of the scaffold has a chemistry that promotes attachment, proliferation, and differentiation of cells. Fourth, the scaffold material displays bioresorbability or biodegradability that can be adjusted to match the rate of cell and tissue growth. Fifth, the scaffold is porous with architecture of interconnected pores that enable growth of cells and tissues into the scaffold and permit adequate transport of nutrients and oxygen. It was found that a porosity greater than 90% is preferable for bone tissue scaffolds (Hu *et al.*, 2002) and the ideal range of pore diameters for bone scaffolds of 100-350 μm has been suggested (Hollinger *et al.*, 1996).

2.4.2 The Processes to Fabricate Porous Scaffolds

Polymeric scaffolds have been processed into porous structures by many methods as following. Moreover, some fabrication techniques involve a combination of these methods.

2.4.2.1 Phase Transition

There are many phase transition techniques used to fabricate porous scaffolds including solvent evaporation (Go golewski *et al.*, 1983), phase separation (Lo, 1996; Gutsche, 1996), freeze drying emulsions (Whang *et al.*, 1995), and gel casting (Coombes *et al.*, 1992).

2.4.2.2 Phase Transition with Leachable Porogens

Phase transition with leachable porogens is widely used to fabricate porous scaffolds. This process was used with compression molding (Mooney *et al.*, 1996), gas foaming (Nam *et al.*, 2000), and solution casting/particulate leaching (Hu, 2002; Goldstein, 1999).

2.4.2.3 Rapid Prototyping

Porous scaffolds can be fabricated from rapid prototyping technique, for example, stereolithography (Cooke *et al.*, 2003) and fused deposition modeling (FDM) (Zein *et al.*, 2002).

2.4.2.4 Fiber Deposition

Porous structures in form of fiber mats are also of interest, which can be fabricated from bonded fiber meshes (Mikos *et al.*, 1993) and electrospinning technique (Li, 2005; Dai, 2005; Coombes, 2004).

2.4.3 Potential of Electrospun (e-spun) Fibers in Scaffolding Application

Laurencin *et al.* (1999) found that cells can attach and organize well around fibers with diameter smaller than the diameter of cells. Therefore, it is a concept to generate the template or scaffold in form of nanofibrous network which mimic the natural ECM and are preferable for cell attachment. Electrospinning is the well known method which nanofibers can be produced. The important advantages of e-spun fibers are the very high surface area-to-volume or mass ratio, high porosity of the e-spun mats that could promote better cell incorporation, and the morphology and size of the fibers that can be easily controlled. Recently, many research pay attention to fabricate e-spun nano-to-micro fibers for using as tissue scaffolds.

Many research reported that cultured cells exhibited a normal phenotype with evidence of filopodia or microvilli on e-spun fibrous scaffolds. Fibrous substrates showed better cell attachment and proliferation than planar structure such as cast films (Bhattarai, 2005; Xu, 2004) and tissue polystyrene plate (TCPS) (Li *et al.*, 2005) and fibrous structure also provided higher uniformity of cells (Bhattarai *et al.*, 2005). It could be due to the greater surface area available for cell attachment and incorporation.

2.4.4 PCL, PLA, PGA, and their Copolymers as the Materials in Bone Scaffolding Application

The materials used for tissue culture may be categorized into two types; natural biopolymer (e.g. chitin, chitosan, collagen, elastin, gelatin, and silk fibroin) and synthetic biopolymer (e.g. PCL, PLA, PGA, and their copolymers). However, the disadvantages of using the natural biopolymer are the limitation and inconsistency of their sources.

Successful culture of osteoblasts and mesenchymal stem cells in osteogenic conditions on e-spun PCL, PLA, and PLGA were reported (Badami, 2006; Yoshimoto, 2003; Li, 2005; Xin, 2007) which the potential to be used in bone tissue engineering was revealed.

E-spun PLLA, PDLA, Poly(ethylene glycol-*co*-DL-lactide) (PEG-PDLLA), and Poly(ethylene glycol-*co*-L-lactide) (PEG-PLLA) substrates were investigated in cultures of MC3T3-E1 cells in the osteogenic conditions (Badami *et al.*, 2006). The cell density after 7 and 14 days of culture on fiber surfaces was comparable to or higher than spin-coated controls, and ALP activity after 14 days was comparable.

Li *et al.* (2005) reported that human mesenchymal stem cells (hMSCs) cultured on e-spun PCL with osteogenic media expressed bone specific proteins (i.e. ALP, bone sialoprotein, and osteocalcin).

Yoshimoto *et al.* (2003) showed that mineralization and type I collagen were observed at 4 weeks of culture of neonatal rat mesenchymal stem cells (MSCs) on e-spun PCL.

E-spun PCL composites were also investigated in culture of bone-marrow stromal cell (BMSC) (Zhang *et al.*, 2005) and human osteoblasts (Fujihara *et al.*, 2005). The BMSCs attached and grew well on the surface of e-spun gelatin/PCL composite scaffold and migrated inside the scaffold up to 114 μm within 1 week of culture (Zhang *et al.*, 2005). Fujihara *et al.* (2005) reported that osteoblasts cultured on e-spun PCL/$CaCO_3$ showed increasing of cell attachment during 5 days of culture.

Not only electrospinning process, PLA, PGA, PCL, and their copolymers scaffolds fabricated by other techniques were widely studied in culture of osteoblasts. Human osteoblasts (HOB) exhibited differing rates of spreading and

morphology when cultured on the substrates with different surface topography. HOB cells were found to be spread to a larger extent on microporous PCL and PCL/HAp, from precipitation casting, than on PCL films (Coombes *et al.*, 2004) at 4 h of culture.

PCL and PCL/HAp scaffolds from fused deposition modeling (FDM) were investigated *in vitro* with human calvarial osteoblasts and *in vivo* of a subcutaneous nude mouse (Chim *et al.*, 2006). Both PCL and PCL/HAp demonstrated tissue growth and mineralization throughout the implants. The biocompatibility of PCL was revealed in which the implants were well integrated into the surrounding tissue and there was no evidence of encapsulation or fibrosis.

Many published works reported on the success of using PLGA as a material for bone scaffolds (Shea, 2000; Karp, 2003; Sedrakyan, 2006). Karp *et al.* (2003) reported *in vitro* bone growth on 2D and 3D PLGA films and the formation of a mineralization matrix.

Osteoblast-like cells exhibited differing rates of mineralization when grown on different polymer surfaces (Calvert, 2000, 2005). In their first report, Calvert *et al.* (2000) evaluated the ability of PCL, PLGA, and some of their blends in the form of thin films in supporting proliferation and differentiation of BMSCs. There was no statistical difference in the proliferation rate of the cells on any substrate at the end of 2 weeks after cell culture. However, PCL was the only material that showed negative staining for Von Kossa (i.e. calcification activities) and ALP. In 2005, working on a different cell line, MC3T3-E1, Calvert *el al.* showed in their subsequent report that, at 6 weeks after cell culture, while there was no significant difference in the osteocalcin activity between the cells grown on PCL and PLGA films, PCL showed much less ALP activity and mineralization of the cells.

2.4.5 Hydroxyapatite (HAp) as a Bioactive Agent in Culture of Bone Cells

For the design of materials in bone scaffolding application in term of biological functions, many researchers made attempt to enhance osteoconductivity of scaffold materials by incorporation of calcium carbonate ($CaCO_3$) or a type of calcium phosphate such as hydroxyapatite (HAp). PCL composite with $CaCO_3$ was investigated in culture of osteoblasts. PCL composite with higher $CaCO_3$ exhibited

more granules and mineralization of cultured cells (Fujihara *et al.*, 2005). HAp, a synthetic calcium phosphate ceramic that mimics the natural apatite composition of bone and teeth, was the most interesting compound to be used as substitute or substrate in bone scaffolding application. Since inorganic phase of bone consists of mainly calcium phosphate compounds in form of HAp, HAp is considered to be biocompatible and osteoconductive. There were many reports found that the presence of HAp help improve the proliferation and differentiation (as expressed by the ALP activity and bone specific proteins) of osteoblasts (Dalby, 2001, 2002; Ma, 2001; Di Silvio, 2002; Kong, 2005; Causa, 2006; Zhao, 2006; Tanaka, 2007).

Tanaka *et al.* (2007) cultured rat marrow mesenchymal cells on the nano-HAp/silk fibroin (HAp-SF) and bare SF sheets. The viable cells after 1 h of culture on the HAp-SF sheet were higher that that on the SF sheet. The ALP activity and osteocalcin secretion of cells on HAp-SF sheet were higher than were those on SF sheet.

Kong *et al.* (2005) showed that the proliferation and the ALP activity of MG63, human osteosarcoma, cells cultured on HAp added zirconia-alumina (ZA) nano-composite gradually increased as the amount of HAp increased.

Ma *et al.* (2001) demonstrated that the proliferation of osteoblasts on PLLA/HAp composite scaffold was higher than PLLA scaffold during 6 weeks in culture. Investigation of mRNA expression determined that bone sialoprotein and osteocalcin of cells cultured on PLLA/HAp were higher than in the PLLA scaffold.

Zhao *et al.* (2006) demonstrated that 3D HAp/chitosan gelatin (HCG) scaffold promoted protein and calcium adsorption in the culture media. Higher proliferation rate and the ALP activity of hMSCs cultured on HCG were observed in comparison with chitosan gelatin (CG) scaffold.

HAp was reported as a highly absorbent material that was shown to strongly adsorb fibronectin and vitronectin, integrin of cell adhesion, from serum, that promote binding of MSC and osteoblast cells to HAp (Kotobuki, 2005; Kilpadi, 2001). The increasing focal contact formation of osteoblasts was also observed with increasing HAp volume (Dalby *et al.*, 2002).

It is though that the protein and calcium ion adsorption of HAp from serum, in which the microenvironment favored for ECM synthesis were formed on

the scaffold surfaces, initiate the preferential surfaces for cell adhesion and thus higher cell proliferation and osteogenic differentiation (Dalby, 2001; Zhao, 2006). For the long-term reaction of osteoblasts in direct contact with HAp, Dalby *et al.* (1999) showed the crystalline deposition within HAp apposed cell layers and described that osteoblasts are either entrapping the HAp particles, or are producing crystalline particles in response to HAp.