CHAPTER II
LITERATURE REVIEW

2.1 Bone Regeneration

Bone is a remarkable organ playing key roles in critical function in human physiology including protection, movement and support of other critical organs, blood production, mineral storage and homeostasis, blood pH regulation, multiple progenitor cell (mesenchymal, hemopoietic) housing, and others. The importance of bone becomes clear in the case of diseases such as osteogenesis imperfecta, osteoarthritis, osteomyelitis, and osteoporosis in which bone does not function properly. These diseases along with traumatic injury, orthopedic surgeries (i.e., total joint arthroplasty, spine arthrodesis, implant fixation, etc.) and primary tumor resections lead to or induce bone defects or voids. The treatment for the critical-sized defects in bone has motivated the development of a wide variety of sophisticated synthetic (tissue-engineered) bone scaffolds (Porter *et al.*, 2009).

Bone tissue engineering provided new medical therapy as an alternative to conventional transplantation methods using polymeric biomaterials with or without bioactive materials such as bioceramics, bioactive glasses or glass ceramics. Applying bioactive materials throughout the composite scaffold matrices can drastically improve the bone regeneration ability of these materials. In addition, various composite scaffolds can be fabricated with blends of synthetic and natural polymers by choice of suitable solvents and preparation methods (Kim *et al.*, 2010). The composite scaffolds that blend synthetic and natural polymers can exploit the advantages of both polymer types (Venugopal, 2010; Choi, 2008).

2.1.1 Bioactive Ceramics

2.1.1.1 Bioactive Glasses and Glass-Ceramics

The base components in most bioactive glasses and glass ceramics, made by traditional high temperature melting, casting and sintering, are SiO_2, Na_2O, CaO, and P_2O_5. With these chemical components, biocompatibility known as bonding to bone was enhanced for the bioactive glasses (Bang *et al.*,

2008). Glass ceramics of the ternary CaO-MgO-SiO_2 system have been reported as good candidate materials for wear resistance, biomedical, and ceramic-coating applications due to their good mechanical and chemical properties (Hill, 2004; Agathopoulos, 2006).

2.1.1.2 Calcium Phosphate Ceramics

Calcium phosphate ceramics, especially tricalcium phosphate (TCP: $Ca_3(PO_4)_2$) and hydroxyapatite (HAp: $Ca_{10}(PO_4)_6(OH)_2$), have been widely studied for clinical uses. TCP and HAp can be prepared using several different methods, such as precipitation method (Lee *et al.*, 2003), hydrolysis method (Nakahira *et al.*, 1999), solid-state reaction (Guo *et al.*, 2004), hydrothermal reactions (Liu *et al.*, 2003) and the sol-gel method (Bose *et al.*, 2003).

HAp is a well known material for filling bone defects. It has been used for skeletal tissue engineering because of its biocompatibility and osteoconductivity. The interconnected porous structure, good mechanical properties, and biocompatibility of biodegradable composite provide a suitable microenvironment to promote osteoblast proliferation and osteogenesis. HAp has no carcinogenic properties and does not cause trigger on allergic reactions (Porter *et al.*, 2004). For bone regeneration, Matsumoto *et al.* (2004) proposed that it is very important to regulate the HAp resorption in the body and to control the release of growth factor proteins at the optimal time and amount. Therefore, they fabricated porous HAp particles as a controlled release carrier, which can regulate the combined amount and the release of cytochrome c (a model protein) at different temperatures. Wang *et al.* (2007) also successfully synthesized nanosized HAp as a controlled release carrier of protein with the study of the adsorptive properties of protein on HAp and different influence parameters such as pH, calcium, and phosphate concentrations during the adsorption process. Ovalbumin (OVA) was used as a model growth factor protein.

2.1.2 Bone Proteins

Bone is a rich source of several molecules including growth factors, chemotactic and attachment factors such as collagen (COL), fibronectin (FN), bone sialoprotein (BSP), and osteopontin (OPN).

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 Fibronectin

Fibronectin, a large molecule of 480 kDa, is mostly found in the liver. It is a dimer that contains one or more of the following three alternatively spliced domains [extra-domain-A (EDA), extra-domain-B (EDB), and the variable region]. Fibronectin itself is required by the osteoblasts to form mineralized nodules in vitro (Moursi *et al.*, 1997). It classically has been viewed as two separate entities: (1) The soluble form is called *Plasma Fibronectin*, is produced by hepatocytes in the liver and circulates in the bloodstream, and contains neither the EDA nor the EDB, but in one of its two chains the variable region is present in its complete length, and (2) the cellular form is produced by a variety of cells, presumably contains one or more of the alternatively spliced domains, and gets incorporated into the matrix (Hynes, 1990; Matsuura, 1988). Even though the soluble plasma fibronectin always was used in the studies of collagen matrix assembly, it has always been assumed that the cellular forms of fibronectin are the ones that are operative in the matrix in vivo (Hynes *et al.*, 1990).

2.1.2.3 Bone Sialoprotein

Bone sialoprotein is an Arg-Gly-Asp (RGD)-containing adhesive protein that is highly conserved, with expression essentially restricted to mineralized tissues. Bone sialoprotein or its transcript has been used as a marker for osteoblast differentiation. It has numerous postulated functions including binding to collagen, promoting mineralization, enhancing osteoprogenitor cell differentiation and attachment and angiogenesis, properties critically relevant to bone formation and repair (Mauney *et al.*, 2005). In vivo studies have demonstrated the efficacy of the bone sialoprotein in promoting bone and tooth repair (Goldberg *et al.*, 2008).

2.1.2.4 Osteopontin

Osteopontin, a glycoprotein with a glycine–arginine–glycine–aspartate–serine (GRGDS) cell- binding sequence, has an attachment capacity that promotes the attraction and distribution of bone formation cells. The concentration of the osteopontin reaches its maximum as a function of stage of mineralized tissue formation (Chen, 1992; Goldberg, 2001).

2.2 Biodegradable Polymers

2.2.1 Poly(ε-caprolactone) (PCL)

PCL, a semi-crystalline aliphatic polyester, is of great interest as it can be obtained by ring-opening polymerization (ROP) (see Figure 2.1) of a relatively cheap monomeric unit "ε -caprolactone" and is known for its extremely low Tg (-60°C) and long-term degradation properties (>24 months to lose total mass). The low melting-point makes the material suited for composting as a means of disposal, due to the temperature obtained during composting routinely exceeding 60°C (Ayfer *et al.*, 2005). PCL is an attractive polymer to use based on its elastomeric properties and high elongation (Schindler *et al.*, 1977). For biomedical applications, PCL has been approved for use by the FDA since the 1970s and can be found in many common sutures and suture components. Specifically with PCL, some of its attractive qualities are its enhanced solubility in organic solvents, ability to be processed at low temperatures, and its non-toxic degradation byproducts. One of its most attractive qualities for use in biomedical applications is its slow rate of degradation. PCL

degrades by hydrolysis and will lose about 50% of its strength in 8 weeks using an *in vitro* degradation test (Yoon *et al.*, 2005). PCL has been blended with several other polymers, including polylactide (PLA), polyglycolic acid (PGA), and polyhydroxybutyrate (PHB) and has been used in many studies relating to biomedical applications (Armstrong, 2010; Aghdam, 2012; La Cara, 2003).

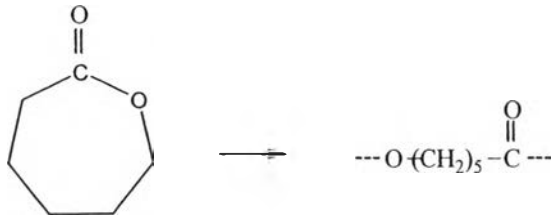

Figure 2.1 The reaction of ring opening polymerization of ε-caprolactone rings.

2.2.2 Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV)

PHBV, a biodegradable and biocompatible polyester, is synthesized by microorganisms by consuming sugars in the presence of propionic acid or produced directly from plants (Mohanty *et al.*, 2000). The chemical structure of PHBV is shown in Figure 2.2. PHBV can be used for biomedical applications due to their biocompatibility and non-toxicity to living tissues (Hocking *et al.*, 1994). In 2008, Miao *et al.* studied the phase behavior and crystallinity of biodegradable PHBV/poly(ethylene succinate) (PES) blends. The results showed that the PHBV and PES were immiscible and the degree of PHBV crystallinity decreased with the addition of PES whereas the degree of PES crystallinity remained unchanged at various blend compositions. Chun and Kim (2000) investigated the thermal properties of biodegradable PHBV/PCL blends and reported that with the addition of PCL, the PHBV crystallization rate in PHBV/PCL blend decreased compared to that of neat PHBV indicating that PCL suppressed the nucleation of PHBV in the PHBV/PCL blend.

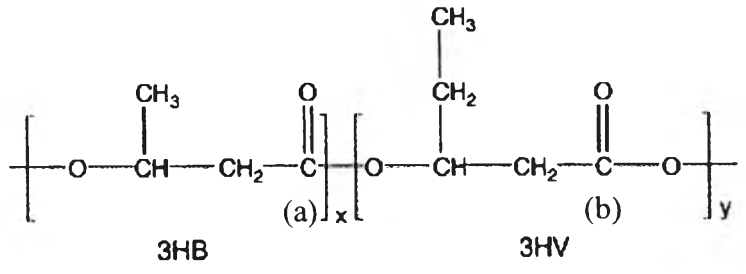

Figure 2.2 Chemical structure of PHBV.

2.3 Electrospinning Process

Electrospining 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.

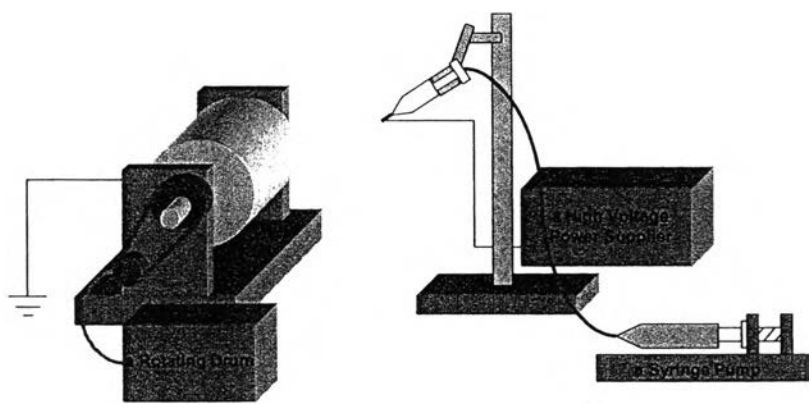

Figure 2.3 A schematic drawing of the electrospinning apparatus.

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

2.3.1 The Initial of 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

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

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 of 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 Fibrous Scaffolds

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 ingrowths, 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 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 (Gogolewski *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 Use of Electrospun Fibrous Scaffolds

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 electrospun fibers are the very high surface area-to-volume or mass ratio, high porosity of the electrospun 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 electrospun 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.

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, PHBV, and their copolymers). However, the disadvantages of using the natural biopolymer are the limitation and inconsistency of their sources.

Li *et al.* (2005) reported that human mesenchymal stem cells (hMSCs) cultured on electrospun 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 electrospun PCL.

Electrospun 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 electrospun 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 electrospun PCL/$CaCO_3$ showed increasing of cell attachment during 5 days 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.

Carbonate hydroxyapatite (CHAp) incorporated in PHBV fibrous scaffolds were also formed through electrospinning (Tong *et al.*, 2011). The CHAp/PHBV nanocomposite scaffolds were found to enhance ALP activity expressed by osteoblastic cells.