CHAPTER II

LITERATURE REVIEW

2.1 Tissue Engineering

Tissue Engineering is an emerging interdisciplinary field that applies the principles of biology and engineering to repair tissue defect and regenerate new tissue and maintain the function of human tissues. There are many approaches to tissue engineering, but all involve one or more of the following key ingredients: harvested cells, recombinant signaling molecules, and three-dimensional (3D) matrices. Tissue engineering for bone typically involves coupling osteogenic cells and/or osteoinductive growth factors with osteoconductive scaffold (Chu *et al.*, 2007).

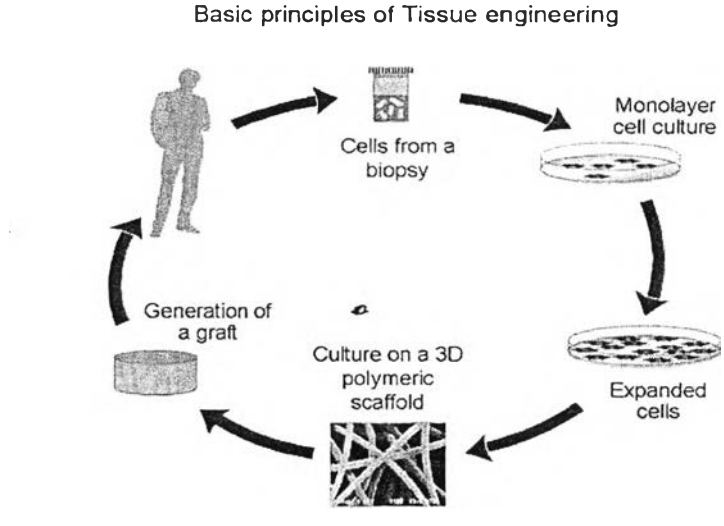

Figure 2.1 Scaffold-guided tissue regeneration.

The structural cues involve the interaction of cells with their extracellular matrix (ECM). The ECM is the part of our body which gives its form and shape. For bone tissue engineering, bone cells (osteoblasts, osteoclasts, osteocytes) exist in a symbiotic relationship with the ECM. Tissue engineering techniques, as demonstrated in Figure 2.1, thus involve mimicking the natural behavior by placing

the cells and growth factors in synthetic scaffolds that act as temporary ECMs. One of the goals of tissue engineering is to develop method to produce the biological substitutes that will restore, maintain or even improve tissue or organ functions.

The ideal scaffolds for tissue engineering should meet several design criteria: (i) The surface should be biocompatible to allow cell growth, attachment, proliferation and differentiation without eliciting an immune response due to toxicity of the material. (ii) The scaffolds should be three dimensional structures with high porosity, high surface to volume and an interconnected pore network for cell in-growth and the flow transportation of nutrients and metabolic waste through and out of the scaffolds (iii) The scaffold should be biodegradable with controllable rate and eventually eliminated or degraded by enzymes and/or circulating biological fluid. (iv) The material should be reproducibly processable into three-dimensional structure (v) The surface chemistry of the scaffold should be suitable to induce cell attachment, proliferation and further differentiation; and (vi) The design of bone tissue engineering scaffolds should maintain appropriate mechanical stability at the site of implantation (*In vivo*) (Hutmacher et al., 2000). 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).

Several materials have been used or synthesized and fabricated into scaffolds for tissue engineering approach. Synthetic and natural inorganic ceramic (e.g. hydroxyapatite and tricalcium phosphate) have been investigated as candidate scaffold material for bone tissue engineering (Burg *et al.*, 2000). This is because these ceramics is exactly like the natural inorganic component of bone and have osteoconductive properties (Le, Geros., 2002). However, these ceramics are intrinsically brittle and cannot match the mechanical properties of bone. Moreover, ceramic scaffolds cannot be expected to be appropriate for the growth of soft tissues (e.g. heart muscle tissue) considering that these tissues possess different cellular receptors and mechanical property requirements. It should be considered that bone is a composite consisting a polymer matrix reinforced with ceramic particles, which polymer is the protein collagen, 30%, and hydroxyapatite (HA), 70% . Therefore,

synthetic and natural polymers are an attractive alternative and versatile in their applications to the growth of most tissues (Sachlos *et al*, 2003).

Most natural polymers used in bone tissue engineering (such as collagen, fibrin, alginate, silk, hyaluronic acid, and chitosan) are biocompatible, biodegradable and solubilized in physiological fluid. However, their drawbacks are immunogenicity, difficulty in processing, and a potential risk of transmitting animal-originated pathogens. In addition, natural polymers have high molecular weight, which make it difficult to process (Lee, S-H., and Shin, H., 2007). Many researchers are interested in synthetic polymer for use as tissue engineering substrate instead of natural polymer because it indeed provides excellent chemical and mechanical properties that natural polymers usually fail to possess. Synthetic polymers provide less danger of immunogenicity and the great advantage of synthetic polymer is its easy processibility with controllable properties such as mechanical strength and chemical properties. The most widely used synthetic polymer is polyester-type polymer such as poly(L-lactic acid), poly(glycolic acid), poly(ε-caprolactone), poly(L-lactide), polyglycolide and their copolymer (poly(lactide-co-glycolide)) since they possess good mechanical property, low immunogenicity, non-toxicity and biodegradable with an adjustable degradation rate. Degradation of these synthetic polymers may occur via hydrolytic pathway and enzymatic cleavage of ester chain in polyesters into water soluble oligomers and monomers that can be eliminated from the implant site. In addition, the degradation mechanism and by-product are not affect the surrounding cells, activating the immune response that causes the inflammation or toxicity. Table 2.1 demonstrates the example of polyester-type polymer in many type of forms and preparation methods for tissue engineering application.

Table 2.1 Polyester-type polymer in many type of forms and preparation methods used for tissue engineering application

Carrier material	Growth Factor	Matrix type	Combination method	Animal model	Reference
PLGA	rhBMP-2	Microsphere	Suspension	Rat	(Woo *et al.*, 2001)
PLGA	IGF-1	Microsphere	Double emulsion /sovent evaporation	Sheep	(Meinel *et al.*, 2003)
Heparin-PLGA	BMP-2	2-D film and 3-D scaffold	Impregnation	BMSCs from rabbit	(Huang *et al.*, 2004)
PLGA	BMP-2	Scaffold	Mixing	Rat(female)	(Jeon *et al.*, 2007)
PLGA	rhBMP-7	Nanosphere in PLLA scaffold	Double emulsion technique encapsulation	Rat (male)	(Wei *et al.*, 2007)
PLGA	BMP-2	Microsphere embedded in gelatin hydrogel	-	Rat	(Kempen *et al.*, 2008)
PLGA	rhBMP-7 and rhIGF-1	Microsphere	Microencapsulation	hMSC	(Wang *et al.*, 2009)
PLGA	rhBMP-2	Scaffold	Plasma treatment	OCT-1 osteoblast-like cell from mouse calvarias	(Shen *et al.*, 2009)
PLGA	IGF-1	Microparticle embedded into silk fibroin scaffold	Encapsulation	-	(Wenk *et al.*, 2009)
PLA-DX-PEG	rhBMP-2	Copolymer	Mixing	Mice (muscle)	(Yoneda *et al.*, 2005)
PLA-PEG	rhBMP-2	Copolymer	Mixing	Rat	(Saito *et al.*, 2003)
PDLLA	IGF-1 and TGF-β1	Thin film coated on the wire	Mixing	Rat (tibia)	(Sumner *et al.*, 2001)
PCL	BMP-4	Scaffold	Injection	Rabbit	(Savarino *et al.*, 2007)

2.2 Basic Bone Biology

Bone is a highly specialized support tissue which is characterized by its rigidity and hardness. It has a unique capability of self-regenerating or self-remodeling to a certain extent throughout the life without leaving a scar (Wutticharoenmongkol, P., 2007). The major functions of bone are:(1) to provide structural support for the body (2) to provide protection of vital organs (3) to provide an environment for marrow (both blood forming and fat storage) (4) to act as a mineral reservoir for calcium and phosphate homeostasis in the body. Bone is made up of support cells (osteoblasts and osteocytes), remodeling cells (osteoclasts) , non-mineral matrix of collageneous and noncollageneous proteins (osteoid) and inorganic mineral salts deposited within the matrix. The descriptions of each composition are follows and shown in Figure 2.2.

2.2.1 Bone Cells

Three distinctly different cell types can be found within bone: the matrix-producing osteoblast, the tissue-resorbing osteoclast, and the osteocyte, which accounts for 90% of all cells in the adult skeleton. (Sommerfeldt *et al.*2001)

Osteoblasts which are the cells derived from mesenchymal stem cells are responsible for bone matrix synthesis and its subsequent mineralization. In the adult skeleton, the majority of bone surfaces that are undergoing neither formation nor resorption (i.e., not being remodeled) are lined by bone lining cells (the inactive form of the osteoblast) and produce osteoid. Osteoid is non-mineral matrix comprised of type I collagen (~94%) and noncollagenous proteins. The hardness and rigidity of bone is due to the presence of mineral salt in the osteoid matrix, which is a crystalline complex of calcium and phosphate (hydroxyapatite).

Osteocyte cells are osteoblasts that become incorporated within the newly formed osteoid which eventually becomes calcified bone. Osteocytes situated deep in bone matrix maintain contact with newly incorporated osteocytes in osteoid, and with osteoblasts and bone lining cells on the bone surfaces, through an extensive network of cell processes (canaliculi). They are thought to be ideally situated to respond to

changes in physical forces upon bone and to transduce messages to the osteoblastic cells on the bone surface, directing them to initiate resorption or formation responses. **Osteoclasts** are cells with large multinucleus , like macrophages, derived from the hematopoietic lineage. Osteoclasts operate in resorption of mineralized tissue and are found attached to the bone surface at sites of active bone resorption. Their characteristic feature is a ruffled edge where active resorption takes place with the secretion of bone-resorbing enzymes, which digest bone matrix (Simon,S.R, 1994).

2.2.2 Extracellular Matrix

The extracellular matrix (ECM) is a complex structural entity surrounding and supporting cells that are found within mammalian tissues. The ECM is often referred to as the connective tissue. The ECM is composed of 2 major classes of substances:

2.2.2.1 Organic Substance Contains 25% of ECM

It is composed of 2 major components; collageneous proteins and noncollageneous proteins. Collageneous proteins mainly consist of 90% - collagen type I. Collagens are the most abundant proteins found in the animal kingdom. It is the major protein comprising the ECM. Another type is noncollageneous proteins including (1) Glycoproteins such as osteonectin, binding Ca^{2+} and collagen and nucleating hydroxylapatite and osteopontin,constituent of cement line; involved in bone remodeling (Salgado *et al*, 2004) (2) Osteocalcin is low molecular weight protein embedded in the extracellular matrix having important signaling functions (bone morphogeneic proteins, growth factors, cytokines and adhesion molecules) or playing a role during the mineralization process (osteopontin, osteonectin, matrix-glaprotein) (Sommerfeldt *et al*, 2001) , (3) Proteoglycans are complex proteins and long chains of repeating disaccharide units called glycosaminoglycans (GAGs such as hyaluronic acid, chondroitin sulfate, heparin, heparan sulfate). Proteoglycans may also help to trap (Weiner S., and Traub W., 1992) and store within the ECM lipids (ground substance), and (4) γ-carboxyglutamic acid containing proteins (vitamin K dependent).

2.2.2.2 Inorganic Substance Contains 70 % of ECM

It is composed of crystalline complexes of calcium and phosphate (hydroxyapatite). $[Ca_{10}(PO_4)_6(OH)_2]$, a plate-like crystal (20 to 80) nm in length with the thickness (2 to 5) nm. Since it is four times smaller than naturally occurring apatites and less perfect in structure, it is more reactive and soluble and facilitates chemical turnover (Weiner,S., and Traub,W., 1992).

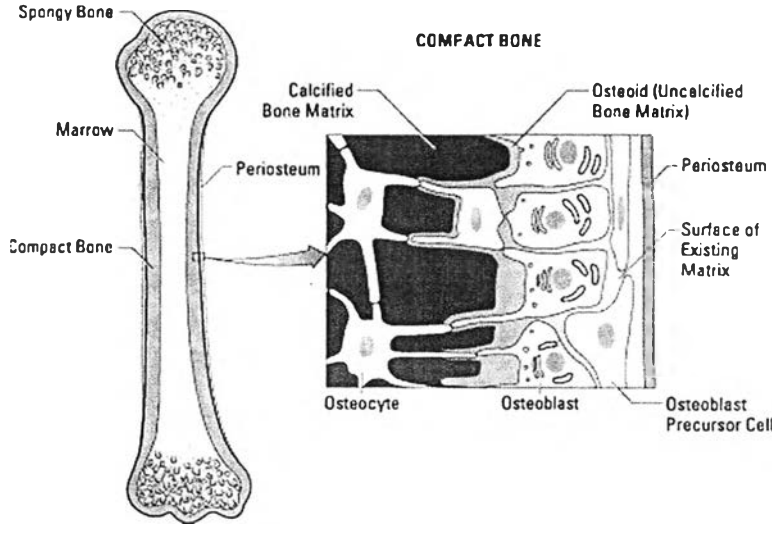

Figure 2.2 Bone matrix.

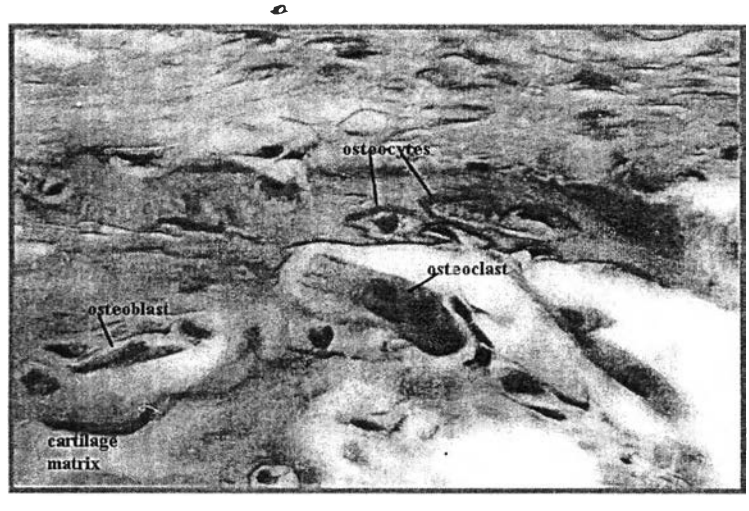

Figure 2.3 Three distinctly different bone cell types.

2.3 Bone Remodeling

Bones are constantly maintained through a process known as remodeling. The two bone remodeling phases are resorption and formation. When equilibrium of bone loss and bone formation is maintained, then a healthy bone state is achieved. However, a disruption of this healthy balance of resorption and formation process can cause the bone to weaken over time due to hormonal changes, injuries, aging or other factors. This remodeling stage is composed of two cells that are responsible for this vital activity of breaking down "old" bone and continuously rebuilding "new" bone. The two cells are osteoblast cells (responsible for adding new collagen and minerals to bones. Osteoblasts are responsible for the second half of skeletal maintenance; these cells are known as the "bone creators.") and osteoclast cells (large cells responsible for removing minerals and collagen from bone. Osteoclasts perform the first step in bone maintenance; they are known as the "bone destroyers."). The entire remodeling cycle for an adult occurs over a three-year period.

2.3.1 Process of Bone Remodeling

The cycle 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. Bone remodeling is accomplished by teams of bone forming cells (osteoblasts) and bone resorbing cells (osteoclasts) that work together in so-called basic multicellular units (BMUs).

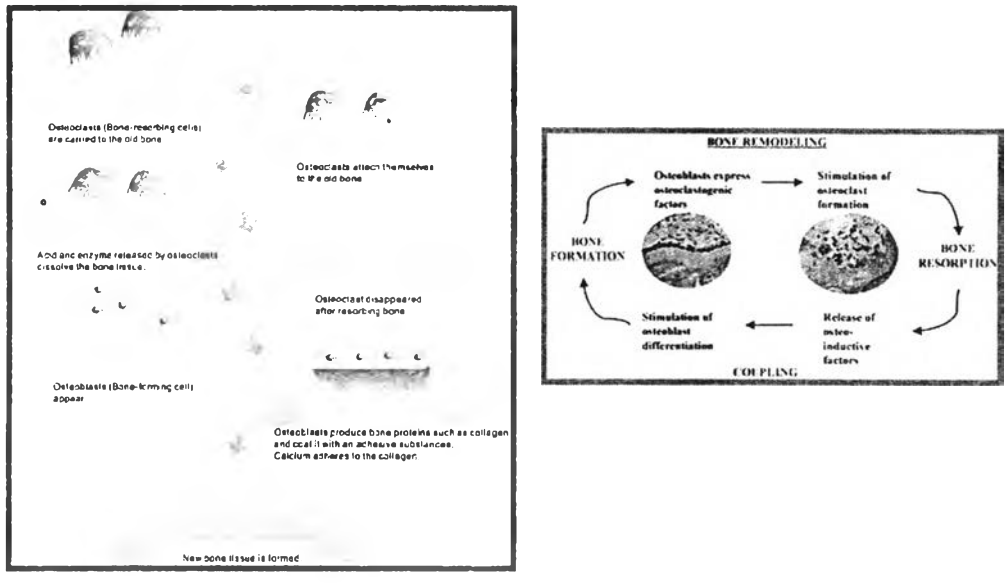

Figure 2.4 Bone remodeling mechanism.

2.3.2 Bone Remodeling and the Osteoclast.

Bone remodeling is a sensitive, dynamic process which begins with a period of osteoclast recruitment and bone resorption. The formation and activity of these large, multinucleated cells is mediated primarily via osteoclastogenic factors and cell-to-cell interactions with various constituents of the bone microenvironment. The inappropriate expression and altered levels of these factors results in the dysregulation of osteoclastogenesis and uncoupling of the bone remodeling process. This frequently leads to the debilitating bone loss associated with tumor-induced bone disease and disorders such as osteoporosis and rheumatoid arthritis.

2.3.3 Bone Remodeling and the Osteoblast.

Osteoblast differentiation and bone formation can be controlled by a number of regulatory molecules. TGF-β for example, is abundant in the bone matrix and is liberated in large quantities following a period of bone resorption. Bone Morphogenetic Proteins (BMPs) are bone-derived molecules of the TGF-β superfamily, which play a clear role in the bone remodeling process and are critical to embryonic development. BMPs have been shown to stimulate osteoblast

differentiation and bone formation along with influencing osteoclast formation and function, although the mechanisms which underlie this process are still emerging.

2.4 Ossification

Ossification is the process of the synthesis of bone from cartilage. There are two types of ossification; intramembranous and endochondral ossification. Bone may be synthesized by intramembranous ossification, endochondral ossification, or a combination of both. Intramembranous ossification is the transformation of the mesenchyme, cells of an embryo into bone. During early development of vertebrate animals, the embryo consists of three primary cell layers: ectoderm on the outside, mesoderm in the middle, and endoderm on the inside. Mesenchyme cells constitute part of the embryo's mesoderm and develop into connective tissue such as bone and blood. The bones of the skull derived directly from mesenchyme cells by intramembranous ossification. Endochondral ossification is the gradual replacement of cartilage by bone during development. This process is responsible for formation of most of the skeleton of vertebrate animals. In this process, actively dividing bone-forming cells (osteoblasts) arise in regions of cartilage called ossification centers. The osteoblasts then develop into osteocytes, which are mature bone cells embedded in the calcified (hardened) part of the bone known as the matrix.

Due to advancement, tissue engineering strategies utilize combination of cells, biodegradable scaffolds, and bioactive molecules (such as proteins, growth factor) to run through natural processes of tissue regeneration and development. One approach in tissue engineering involves the delivery of growth factors from optimally designed biodegradable carriers to stimulate cellular adhesion, proliferation, and differentiation in order to promote bone regeneration. The results from systematic administration of growth factors are often unpredictable, probably due to their short biological half life, lack of long term stability, tissue specificity, and potential dose dependent carcinogenicity. The carrier primarily acts as a local regulator to control doses and kinetics of released growth factor, thus increasing their potential retention time at therapeutic concentration levels recently play an important role. Carriers was extended to serving as a temporary substrate and three-dimensional matrix for

cellular infiltration, in which cells can grow and become particular tissue types in concert with degradation of the carrier material. Toward development of an ideal carrier system for bone regeneration, there have been extensive investigations on material types and their processing conditions. Natural and synthetic polymers, inorganic materials, and their composites have been formulated into porous scaffolds, nanofibrous membranes, microparticles, microsphere, nanosphere and hydrogels. Porous scaffolds are the heart of bone tissue engineering approaches. The features of scaffolds are important for regeneration of bone, which can be prepared by using traditional scaffold manufacturing technologies, such as fiber bonding, solvent casting, solution casting particulate leaching, membrane lamination, hydrothermal, impregnation, gas foaming, phase separation, melt moulding, emulsion freeze drying, freeze drying, and electrospinning fibre meshes/fibre bonding in many forms (microparticle, microsphere, hydrogel, scaffold etc.) Microparticle are particles between 0.1 and 100 μm in size having a much larger surface-to-volume ratio than at the macroscale, and thus their behavior can be quite different. Microsphere refers as small spherical units that can contain protein, growth factors and other substances inside, and can release those sustances over a desired period of time and used as carriers of pharmaceutical substances (Meinel *et al.*2003). Hydrogels are three-dimensionally structured networks of hydrophilic polymers containing a large amount of water, generally more than 50% of the total weight. Hydrogels can be formed through chemical or physical crosslink of polymer. Growth factor can be regulated by controlling the chemical and physical properties of the hydrogels from a few days to several months depending on the tissue type (Lee, K.Y., and Yuk, S.H., 2007). Hydrogel does not always function as a good scaffold of migration, proliferation of cells, because of no porous structure necessary for cell infiltration. A delivery carrier material in bone tissue engineering should meet general design criteria mentioned before; biocompatible, biodegradable, three-dimensional structure with high porosity and so on. Therefore, the methods used to fabricate the scaffolds or the forms of the scaffold are both crucial important to consider.

2.5 Scaffold Preparation Methods

It is important to take into consideration of the method chosen to produce a scaffold which can generate desirable properties of scaffold for tissue engineering , such as porosity, pore size, and mechanical strength and to ensure that the method has no adverse effects on mechanical property or biocompatibility of material. Another consideration is the use of high temperatures and severe chemicals during scaffold preparation which can inactivate the incorporation of bioactive agents (e.g. growth factors, proteins). Several techniques have been developed to process synthetic and natural scaffold materials into porous structures. A description of the different conventional scaffold fabrication techniques follows.

2.5.1 Gas Foaming

A biodegradable polymer, such as PLGA is saturated with carbon dioxide (CO_2) at high pressures (Mooney *et al.*, 1996). The solubility of the gas in the polymer is the decreased rapidly by bringing the CO_2 pressure back to atmospheric level. This results in nucleation and growth of gas bubbles, or cells, with sizes ranging between (100 to 500) μm in the polymer.This technique has been developed to overcome the necessity to use organic solvents and solid porogens. up to 93% and 100 mm of porosities and pore sizes can be obtained using this technique, but the pores are largely unconnected, especially on the surface of the foam, making cell seeding and migration within the foam difficult. Moreover, the high temperatures involved in the disc formation prohibit the incorporation of cells or bioactive molecules and the unconnected pore structure (Mikos *et al.*, 2000).

2.5.2 Gas Foaming/Salt Leaching Method

This technique is based on the idea that sieved salt particles of ammonium bicarbonate dispersed within a polymer–solvent mixture generates ammonia and carbon dioxide gases within solidifying matrices upon contact with hot water, thereby producing highly porous structures. These scaffolds showed macropore structures of over 200 μm with no visible surface skin layer, which permitted sufficient cell seeding within the scaffolds. Porosities could be controlled by the amount of ammonium bicarbonate incorporated to the polymer. Moreover, it

was possible to make various scaffolds with different geometries and sizes by a hand-shaping or molding process because the polymer–salt mixture became a gel paste after a partial evaporation of the solvent (Lim *et al.*, 2008).

2.5.3 Freeze Drying

The process by which water (and other solvents) are removed from a frozen material by sublimation and processes: freezing, primary drying (sublimation), and secondary drying (desorption). Synthetic polymers, such as PLGA, are dissolved in glacial acetic acid or benzene. The resultant solution is then frozen and freeze-dried to yield porous matrices (Hsu *et al.*, 1997).

2.5.4 Emulsification/Freeze-Drying

This technique does not require the use of a solid porogen like solvent-casting particulate-leaching. A synthetic polymer is firstly dissolved into a suitable solvent (e.g. polylactic acid in dichloromethane), water is then added to the polymeric solution and the two liquids are mixed in order to obtain an emulsion. Before the two phases can separate, the emulsion is cast into a mold and quickly frozen by immersion into liquid nitrogen. The frozen emulsion is subsequently freeze-dried to remove the dispersed water and the solvent, then leaving a solidified, porous polymeric structure. While emulsification and freeze-drying allows a faster preparation if compared to solvent-casting particulate-leaching, Although it does not require a time consuming leaching step, it still requires the use of solvents, pore size is relatively small and porosity is often irregular. Moreover, Freeze-drying by itself is also a commonly employed technique for the fabrication of scaffolds.

2.5.5 Thermally Induced Phase Separation (TIPS)

The fundamental concept of this method is similar to the emulsification/freeze-drying technique. Liquid-liquid phase separation employs thermodynamic principles to create polymer-rich and polymer-poor phases within a polymer solution. The polymer poor phase is then removed, leaving a highly porous polymer network.The polymers are dissolved in a solvent with a low melting point and that is easy to volatilize, such as naphthalene, phenol or 1,4 dioxane. In some cases, small amounts of water are added as a non-solvent to induce phase separation. The polymer solution is cooled below the melting point of the solvent (polymer poor phase) and then vacuum dried for several days to insure complete solvent

sublimation. The cooling parameters for the solution play an important role in determining the morphology of the resultant scaffold (Mikos *et al.*, 2000). Liquid-liquid phase separation presents the same drawbacks of emulsification/freeze-drying.

2.5.6 Phase Separation/Emulsification

This method is based on concepts of phase separation. Whang et al. (1995) prepared PLGA by dissolving in methylene chloride and then distilled water is added to form an emulsion. The polymer/water mixture is cast into a mold and quenched by placing in liquid nitrogen. After quenching, the scaffolds are freeze-dried at -55°C, resulting in the removal of the dispersed water and polymer solvents. Scaffolds with large porosities (up to 95%), but small pore sizes (13 to 35) mm have been fabricated using this technique. These parameters are very dependent on many factors such as the ratio of polymer solution to water and viscosity of the emulsion that influence the emulsion's stability prior to quenching. Therefore, with further adjustment, it is possible that pore size could be increased. Although, this technique is advantageous because it does not require an extra washing/leaching step, the use of organic solvents must be taken into account for the incorperation of cells and bioactive molecules. This indicates that this fabrication method currently has limited usefulness in the field of tissue engineering.

2.5.7 Solid Freeform Fabrication

Since most of conventional scaffold fabrication method described approaches are limited when it comes to the control of porosity and pore size, computer assisted design and manufacturing techniques (CAD/ CAM) have been introduced to tissue engineering application. The technology transfer of solid freeform fabrication (SFF) to tissue engineering may be the key to produce scaffolds with customised external shape and predefined and reproducible internal morphology, which not only can control pore size, porosity an pore distribution, but can also make structures to increase the mass transport of oxygen and nutrients throughout the scaffold (Sachlos *et al*, 2003). It is difficult to make customized scaffolds with specially designed functional gradient material (Yu *et al.*, 2007). SFF technologies involve building 3 dimensional objects using layered manufacturing strategies. The general process involves producing a computer-generated model using computer-aided design (CAD) software. This CAD model is then expressed as

a series of cross-sectional layers. The data is then carried out to the SFF machine, which produces the physical model. Starting from the bottom and building layers up, each newly formed layer adheres to the previous. Each layer corresponds to a crosssectional division. Post-processing may be required to remove temporary support structures. Furthermore, data obtained from Computerised Tomography (CT) or Magnetic Resonance Imaging (MRI) medical scans can be used to create a customised CAD model and consequently a scaffold possessing realized by using ink-jet printing of polymer powders or through Fused Deposition Modeling of a polymer melt, the exact external shape required to correct the damaged tissue site.

2.5.8 Electrospinning

In the electrospinning process, a high voltage is used to create an electrically charged jet of polymer solution or melt, which dries or solidifies to leave a polymer fiber . One electrode is placed into the spinning solution/melt and the other attached to a collector. Electric field is subjected to the end of a capillary tube that contains the polymer fluid held by its surface tension. This induces a charge on the surface of the liquid. Mutual charge repulsion causes a force directly opposite to the surface tension. As the intensity of the electric field is increased, the hemispherical surface of the fluid at the tip of the capillary tube elongates to form a conical shape known as the Taylor cone. With increasing field, a critical value is attained when the repulsive electrostatic force overcomes the surface tension and a charged jet of fluid is ejected from the tip of the Taylor cone. The discharged polymer solution jet undergoes a whipping process where in the solvent evaporates, leaving behind a charged polymer fiber, which lays itself randomly on a grounded collecting metal screen. In the case of the melt the discharged jet solidifies when it travels in the air and is collected on the grounded metal screen.

2.5.9 Solvent Casing

Solvent casing, an improved method has been found for making a film by casting a film of a hot concentrated polymer solution in organic solvent onto a cooled surface to provide a film consisting of polymer and volatile solvent, where the film is contacted with aqueous medium to substantially remove the solvent from the film and incorporate water internally (United States Patent 4405550). The process of solvent casting is shown in Figure 2.5.

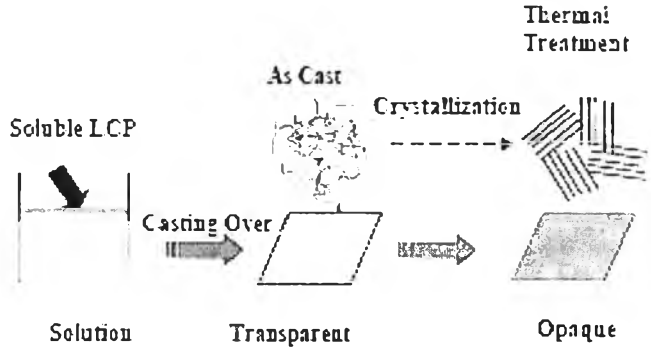

Figure 2.5 Polymer film fabrication using solvent cast film.

2.2.10 Solvent-Casting/Particulate-Leaching

This technique is solvent casting using particulate porogens to form sponge-like scaffolds. SCPL involves the dissolution of the polymer in suitable organic solven mixing with porogen (porogen can be an inorganic salt like sodium chloride, crystals of saccharose, gelatin spheres or paraffin spheres). The size of the porogen particles will affect the size of the scaffold pores, while the polymer to porogen ratio is directly correlated to the amount of porosity of the final structure. The solvent is subsequently evaporated, then the composite structure in the mold is immersed in a bath of a liquid suitable for dissolving the porogen (e.g. water in case of sodium chloride, saccharose and gelatin or an aliphatic solvent like hexane for paraffin). Once the porogen has been fully dissolved a porous structure is obtained. The main advantages of this technique are the ease of processing without the need of specialized equipment and the pore size, porosity of the porous materials can be controlled by the porogen particle size and concentration (Mikos *et al.*, 1993, Edwards *et al.*, 2004). On the other hand, the small thickness range that can be obtained. Another drawback of this method is its use of organic solvents which must be fully removed to avoid any possible damage to the cells seeded on the scaffold.

2.6 Tissue Engineering Scaffold Materials

The polymer scaffold in the tissue engineering is designed to mimic many roles of the extracellular matrices in the body. (Lee *et al*, 2007) To guide *in vitro* or *in vivo* tissue regeneration, it is necessary to obtain appropriate scaffold material, which satisfy all the goals required: biocompatibility, sufficient mechanical strength, osteoconductivity, ability to be fabricated into functional shapes easily, no immunogenic potentiality, controlled bioresorbability high interconnected macroporosity (Alam *et al*, 2001), hydrophobicity, etc (Lee *et al*, 2007).

Synthetic polymer such as PLA, PGA, PLGA, PCL, PPF, PEG etc. Polylactone-type biodegradable polymers, such as poly(L-lactide) (PLLA), polyglycolide (PGA) and their copolymer poly(lactide-co-glycolide) (PLGA) are extensively studied as scaffold materials for tissue engineering (Sachlos *et al.*, 2003), since they possess good mechanical property, low immunogenicity, non-toxicity and adjustable degradation rate. Degradation may occur via hydrolytic pathway and enzymatic cleavage into water soluble oligomers and monomers that eliminate from the implantsite. The degradation mechanism process is not significantly affected by the presence of cells.

Calvert *et al.*(2000) performed poly (carprolactone), poly (DL-lacitc co-glycolic acid) (PLGA) and combination of these polymers to create a novel biomaterial for bone tissue engineering. Bone marrow stromal cells were cultured. The result showed that the rate of proliferation was not significantly different for any of the polymers or their combinations indicated that all polymers are candidates for a novel bone tissue engineering scaffold. There was no limit in choice of polymers for new scaffold materials.

In our previous study, Prasansuklarb A. (2008) fabricated bone scaffold materials from various aliphatic polyesters: polycaprolactone (PCL), poly(1,4-butylene succinate) extended with 1,6-diisocyanatohexane (PBSu-DCH), poly(lactic acid) (PLA), poly(3-hydroxybutyric acid) (PHB), and poly(3-hydroxybutyric acid-*co*-hydroxyvaleric acid (PHBV) at a 30:1 NaCl/polymer weight ratio by solvent casting and salt particulate leaching technique. The average pore diameters and porosity created by this technique were in the range of (400 to 500) μm and (93 to

95) %, respectively. The compressive modulus of the scaffolds was decreased in the order of PLA > PHBV ≈ PHB > PBSu-DCH > PCL and the degradation rates of the scaffolds ranked as follows : PBSu-DCH > PCL > PHB > PHBV > PLA. Human osteoblast cells (SaOS-2) were seeded on the PCL and PBSu-DCH scaffolds appeared to attach and proliferate well. Additionally, ALP activity result signified that PCL and PBSu-DCH scaffolds also better promoted the differentiation of SaOS-2 than the others. This indicated that PCL can be an improved alternative to other polymer-base scaffold for tissue engineering applications.

2.7 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).

2.7.1 Polycaprolactone (PCL)

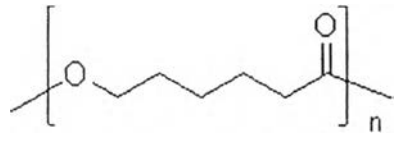

Figure 2.6 The structure of polycaprolactone.

Polycaprolactone (PCL) is a biodegradable polyester with a low melting point of around 60°C and a glass transition temperature of about −60°C. It can be prepared by ring opening polymerization of ε-caprolactone using a catalyst such as stannous octanoate. Polycaprolactone has good water, oil, solvent ,and chlorine resistance.

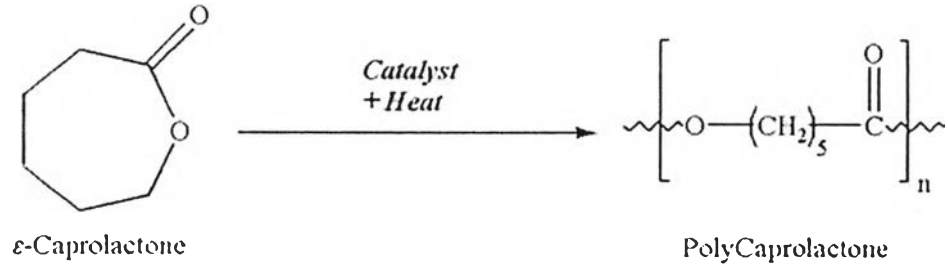

Figure 2.7 Ring opening polymerization of ε-caprolactone to polycaprolactone.

PCL can be degraded by hydrolysis of its ester linkages in physiological conditions (such as in the human body) and therefore has received a great deal of attention for use as an implantable biomaterial. In particular it is especially interesting for the preparation of long term implantable devices, owing to its degradation which is even slower than that of polylactide (Prasansuklarb A., 2008). The structure of PCL is shown in Figure 2.6.

2.7.2 Poly(3-hydroxybutyrate) (PHB)

Polyhydroxybutyrate (PHB) is a polyhydroxyalkanoate (PHA), a polymer belonging to the polyesters class that are of interest as bio-derived and biodegradable plastics. The poly-3-hydroxybutyrate (P3HB) form of PHB is probably the most common type of polyhydroxyalkanoate, but other polymers of this class are produced by a variety of organisms: these include poly-4-hydroxybutyrate (P4HB), polyhydroxyvalerate (PHV), polyhydroxyhexanoate (PHH), polyhydroxyoctanoate (PHO) and their copolymers. (http://en.wikipedia.org/wiki/Polyhydroxybutyrate) Polyhydroxybutyrate (PHB) has therefore received special interest as an implant material, because D,L-b-HB is a

normal component of blood and tissue. (Shan, C. et al., 2006) Poly(3-hydroxybutyrate) (PHB), as a member of polyhydroxyalkanoates (PHA) family, has attracted much attention for a variety of medical applications because of its biodegradation and excellent biocompatibility. (Donghua, G. et al., 2008) PHB is of particular interest for bone tissue application as it was demonstrated to produce a consistent favorable bone tissue adaptation response with no evidence of an undesirable chronic inflammatory response after implantation periods of up to 12 months. Bone is formed close to the material and subsequently becomes highly organized, with up to 80% of the implant surface lying in direct apposition to new bone.(Rezwan, K. et al., 2006)

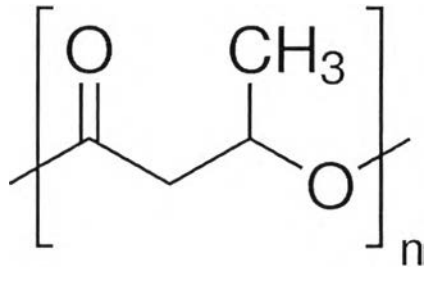

Figure 2.8 The structure of Polyhydroxybutyrate (PHB).

2.7.3 Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV)

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate (PHBV) is a polyhydroxyalkanoate-type polymer. It is biodegradable, nontoxic, biocompatible plastic produced naturally bybacteria and a good alternative for many non-biodegradable synthetic polymers. It is athermoplastic linear aliphatic polyester. PHBV is synthesized by bacteria as storage compounds under growth limiting conditions. It can be produced from glucose and propionate by the recombinant*Escherichia coli* strains. Many other bacteria like *Paracoccus denitrificans* and *Ralstonia eutropha* are also capable of producing it. It can also be synthesized from genetically engineered plants. PHBV is a copolymer of 3-hydroxybutanoic acid and 3-hydroxypentanoic acid. PHBV may also be synthesized

from butyrolactone and valerolactone in the presence of oligomeric aluminoxane as catalyst. (http://en.wikipedia.org/wiki/Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

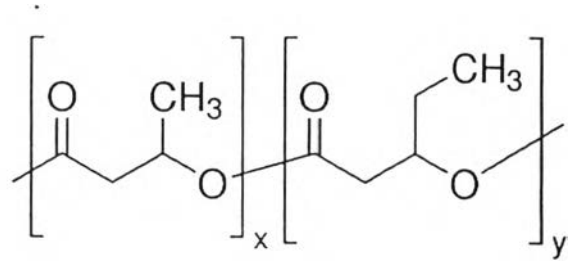

Figure 2.9 The structure of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate (PHBV).

2.7.4 Biodegradation of Polymer

The main factors that determine the rate of degradation are:

2.7.4.1 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).

2.7.4.2 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).

2.7.4.3 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).

2.7.4.4 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).

2.8 Hydroxyapatite (HA)

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 (HA). 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). HA, 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 HA, HA is considered to be biocompatible and osteoconductive. There were many reports found that the presence of HA 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-HA/silk fibroin (HA-SF) and bare SF sheets. The viable cells after 1 h of culture on the HA-SF sheet were higher that that on the SF sheet. The ALP activity and osteocalcin secretion of cells on HA-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 HA added zirconia-alumina (ZA) nano-composite gradually increased as the amount of HA increased.

HA 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 HA (Kotobuki, 2005; Kilpadi,

2001). The increasing focal contact formation of osteoblasts was also observed with increasing HA volume (Dalby *et al.*, 2002).

It is though that the protein and calcium ion adsorption of HA 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 HA, Dalby *et al.* (1999) showed the crystalline deposition within HA apposed cell layers and described that osteoblasts are either entrapping the HA particles, or are producing crystalline particles in response to HA.

Ma *et al.* (2001) demonstrated that the proliferation of osteoblasts on PLLA/HA 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/HA were higher than in the PLLA scaffold.

Zhao *et al.* (2006) demonstrated that 3D HA/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.