CHAPTER II
LITERATURE REVIEW

THEORETICAL BACKGROUND AND 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).

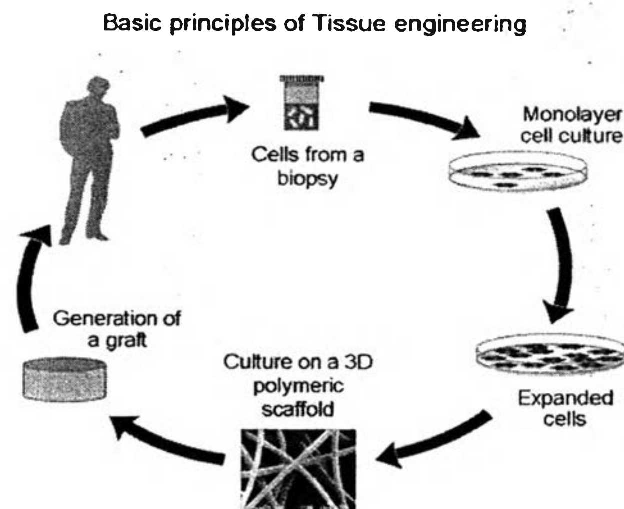

Figure 2.1 Scaffold-guided tissue regeneration.

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,

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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).

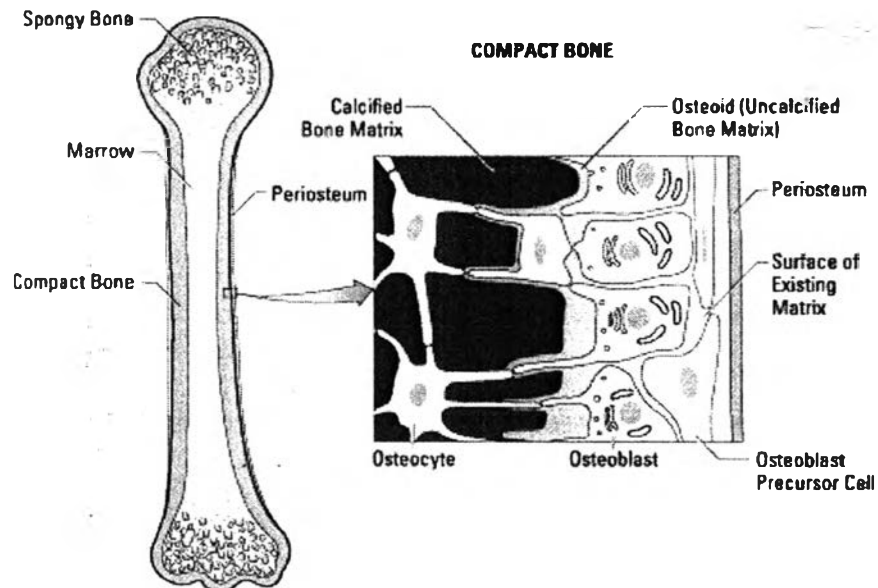

Figure 2.2 Bone matrix.

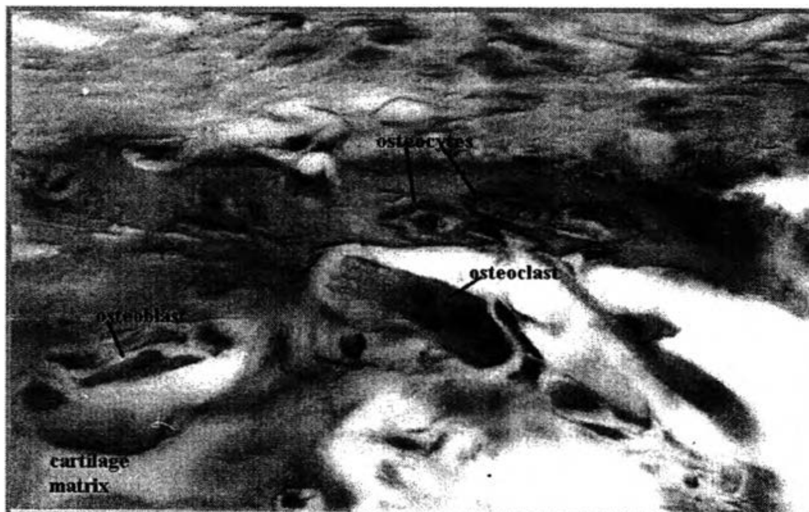

Figure 2.3 Three distinctly different bone cell types. Picture from http://cell.utmb.edu

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).

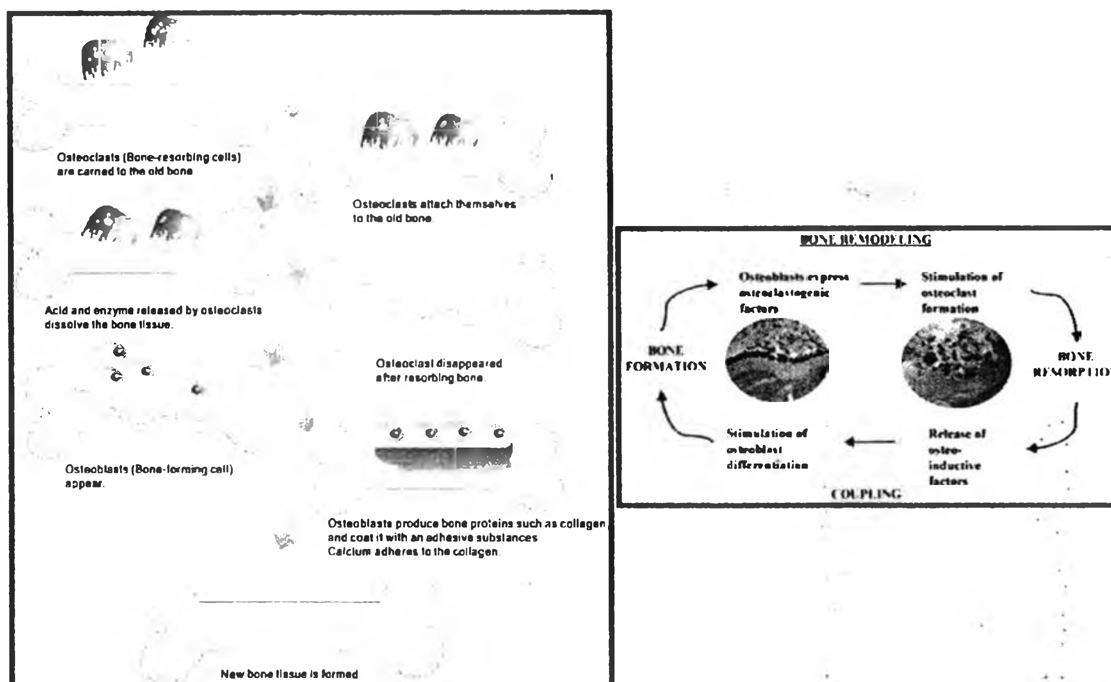

Figure 2.4 Bone remodeling mechanism.

2.3.2 <u>Bone Remodeling and the Osteoclast.</u>

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 <u>Bone Remodeling and the Osteoblast.</u>

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 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.

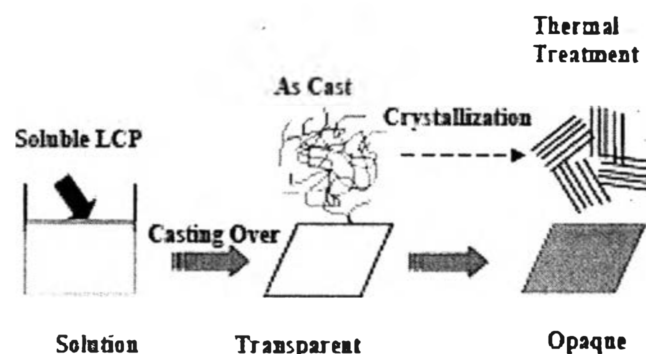

Figure 2.5 Polymer film fabrication using solvent cast film.

2.5.2 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.

2.5.3 <u>Gas Foaming</u>

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).

2.5.4 <u>Gas Foaming/Salt Leaching Method</u>

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).

2.5.5 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).

2.5.6 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.

2.5.7 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.

2.5.8 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.

2.5.9 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 cross-sectional 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.

2.6 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. The schematic of electrospinning process is shown in Figure 2.6.

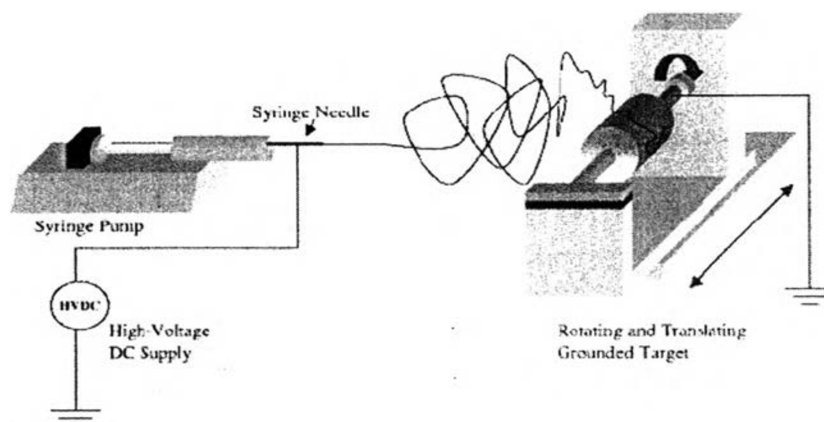

Figure 2.6 The schematic of electrospinning process.

An important characteristic of electrospinning is the ability to make fibers with diameters in the range of manometers to a few microns. Consequently these fibers have a large surface area per unit mass so that nanowoven fabrics of these nanofibers collected on a screen can be used for example, for filtration of submicron particles in separation industries and biomedical applications , such as wound dressing in medical industry, tissue engineering scaffolds and artificial blood vessels. The use of electrospun fibers at critical places in advanced composites to improve crack resistance is also promising. In addition to this, the ultrafine fibres produced by electrospinning are expected to have two main properties, a very high surface to volume ratio, and a relatively defect free structure at the molecular level. This first property makes electrospun material suitable for activities requiring a high degree of physical contact, such as providing sites for chemical reactions, or the capture of small sized particulate material by physical entanglement - filtration. The second property should allow electrospun fibres to approach the theoretical maximum strength of the spun material, opening up the possibility of making high mechanical performance composite materials. Electrospinning is hence being investigated as a source of cost-effective, easy to manufacture wound dressings, medical implants, and scaffolds for the production of artificial human tissues. The above description of the process suggests that the following parameters: solution properties including

viscosity, conductivity, and surface tension; controlled variables including hydrostatic pressure in the capillary, electric potential at the tip, and the distance between the tip and the collection screen; and ambient parameters including temperature, humidity, and air velocity in the electrospinning chamber can affect the process.By appropriately varying one or more of the above parameters, fibers were successfully electrospun from water soluble polymers, biopolymers, and liquid crystalline polymers. These scaffolds fulfill a similar purpose as the extracellular matrix in natural tissue. Biodegradable polymers, such as polycaprolactone, are typically used for this purpose. These fibers may then be coated with collagen to promote cell attachment, although collagen has successfully been spun directly into membrane (Matthews *et al.*, 2002).

Yoshimato *et al.* (2003) prepared microporous, non-woven poly (ε-caprolactone) scaffolds by electrostatic fiber spinning (electrospinning). Mesenchyme stem cells (MSCs) derived from the bone marrow of neonatal rats were cultured, expanded and seeded on electrospun PCL scaffolds. SEM demonstrated that the cell migrated inside the scaffold and produced and extracellular matrix of collagen throughout the scaffold. The cell-polymer constructs maintained the size and shape of the original scaffolds. This suggested that eletrospun PCL is a potential candidate scaffold for bone tissue engineering.

Bolgen *et al.* (2005) prepared non-woven materials from poly(ε-caprolactone) (PCL) by electrospining. Electrospun membrane were more hydrophobic than PCL solvent casted one; therefore, their degradation was a much slower process.

2.7 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

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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.

2.8 Polycaprolactone (PCL)

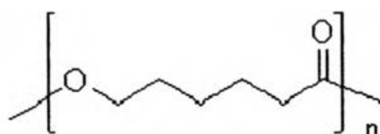

Figure 2.7 The structure of polycaprolactone.

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.

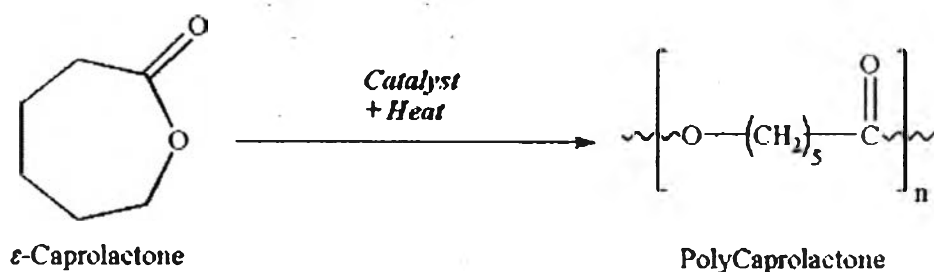

Figure 2.8 Ring opening polymerization of ε-caprolactone to polycaprolactone.

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 2.8.

The our study , MC3T3-E1 murine preosteoblast cell was cultured slide coated with either PLGA or PCL and on uncoated glass slide as control. The MC3T3-E1 cell appeared to grow to confluence at equivalent rates on all three sufaces, glass, PLGA, and PCL. The data from the expression of the genes for alkaline phosphatase and osteocalcin showed that PLGA was more able to support the expression of osteoblast phenotype when compared with PCL. However, because PCL has favorable handing characteristics and strength modifications of PCL may prompt further investigation.

2.9 Surface Modification

The cytocompatibility of the biodegradable polyester-typed scaffold is not good because of the lack of cell recognition sites on their surface resulting from hydrophobicity of non-polar groups along its backbone, so it is far from the ideal scaffold which can bind growth factor on it and make the scaffold supply biological signals for guiding and accelerating cell attachment, migration, differentiation and proliferation. The initial attachment of a cell to a substrate is mediated by cell-surface adhesion factors. Intensive research has shown that cell-extracellular matrix (ECM) interactions participate directly in promoting cell adhesion, migration, growth and differentiation. Various ECM molecules such as type-I collagen, fibrinogen and fibronectin containing specific amino acid receptors that allow them to bind directly to cell-surface receptors. Other studies have also demonstrated the binding functions of the arginine–glycine–aspartic (RGD) peptide sequence in some extracellular matrix molecules. Based on these fundamental findings, studies have been contributed to mimic the extracellular matrix on substrate surfaces, by immobilizing extracellular molecules or other bioactive molecules to the scaffold surface (Hsu *et al.*, 2006). There are several methods to immobilize proteins on a substrate for biomedical and biochemical applications. These can be divided into two general categories; physical and chemical. Various studies have demonstrated that coating the scaffold surface with collagen, or pre-soaking scaffolds in media improves cell seeding efficiency and

spreading (Yang *et al.*, 2003). Physical coating is a simple technique; however, this coating is less biologically stable.

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

- Plasma treatment was investigated to introduce proper functional groups on the surface of a polymer only by selecting and applying some suitable gas. Oxygen plasma treatment provided property for surface of poly(lactide-co-glycolide) (PLGA) matrix for anchoring rhBMP-2 (Shen *et al.*, 2009).
- End-grafting or *in situ* polymerizations by photo or by radio frequency have been developed to alter the surface property of materials by producing hydrophilic layer onto bulk biomedical polymers. Grafting copolymerization of MMA initiated under UV light (PLLA-g-PMMA) to enhance cytocompatibility between materials and biomacromolecules (Ma et al., 2002).
- Aminolysis is another method that has been applied to most biodegradable, aliphatic polyester such as PCL, PLLA. Reaction between ester groups of PLLA and amino groups of hexanediamine produces free amino groups that can further react with functional groups of protein or growth factor to improve its cytocompactibility; cell-polymer interaction. (Zhu et al., 2002).

Guan *et al.* (2001) prepared functional polyurethane (PU) surface by photografting *N,N*-dimethyl-aminoethyl methacrylate (DMAEM) to introduce the amino groups onto the surface. Grafting copolymerization was conducted by the combined

use of the photo-oxidation and irradiation grafting. Human umbilical vein endothelium (HUVE) cells were seeded on the grafted surface cell attachment and proliferation were evaluated. The comparison of PU and DMAEM grafted membrane showed that cell attachment is better on the grafted membrane. It suggested that cell attachment was promoted by the presence of amino groups.

Park *et al.* (2006) prepared biodegradable nanofibrous poly(L-lactic acid) PLLA scaffold by an electrospinning process for use in tissue regeneration. The nanofibrous scaffold was treated with oxygen plasma and then simultaneously *in situ* grafted with hydrophilic acrylic acid (AA) to obtained PLLA-*g*-PAA. The fiber diameter, pore size and porosity of scaffold were estimated as (250 to 750) nm, ~30μm and 95%, respectively. After the fibroblasts were cultured for up to 6 days, cell adhesion and proliferation were much improved on the PLLA-*g*-PAA scaffold than on either PLLA film or unmodified PLLA scaffold. This work demonstrated that the applications of plasma treatment and hydrophilic AA grafting were effective to modify the surface of PLLA scaffold and improved cell adhesion and proliferation.

In general, all the modifications can apparently improve cell response to the substrate surfaces, resulting in an improved cytocompatibility. These methods are not restricted to extracellular matrix molecules and can be extended to other biomacromolecules such as cell growth factors, cell morphology generation proteins, polysaccharides, and so forth.

2.10 General Description of Proteins Immobilized onto PCL Scaffolds

2.10.1 Gelatin

Gelatin is a translucent, colorless, odorless, brittle, nearly tasteless solid substance, derived from the collagen inside animals' skin and bones. It is commonly used as a gelling agent in food, pharmaceuticals, photography, and cosmetic manufacturing. Gelatin is a protein produced by partial hydrolysis of collagen extracted from the bones, connective tissues, organs and some intestines of animals such as domesticated cattle, pigs, and horses. The natural molecular bonds

between individual collagen strands are broken down into a form that rearranges more easily. Gelatin contains many glycine (almost 1 in 3 residues, arranged every third residue), proline and 4-hydroxyproline residues. A typical structure is -Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro- as shown in Figure 2.9.

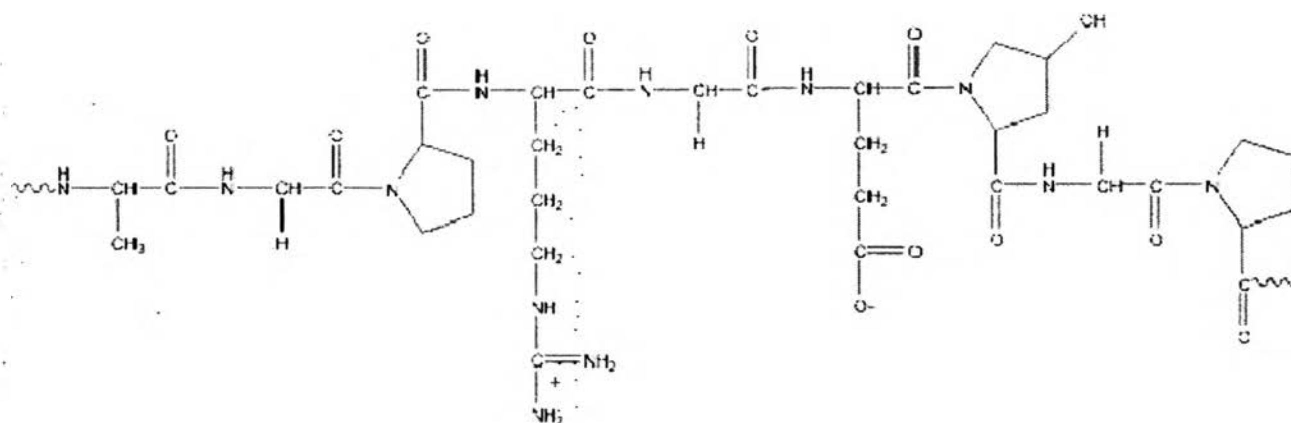

Figure 2.9 The structure of gelatin.

Gelatin melts to a liquid when heated and solidifies when cooled again. Together with water, it forms a semi-solid colloid gel. Gelatin forms a solution of high viscosity in water, which sets to a gel on cooling, and its chemical composition is, in many respects, closely similar to that of its parent collagen (Ward, A.G., and Courts, A. ,1977). Gelatin solutions show viscoelastic flow and streaming birefringence. If gelatin is put into contact with cold water, some of the material dissolves. The solubility of the gelatin is determined by the method of manufacture. Typically, gelatin can be dispersed in a relatively concentrated acid and is also soluble in most polar solvents. The mechanical properties are very sensitive to temperature variations, previous thermal history of the gel, and time. The viscosity of the gelatin/water mixture increases with concentration and when kept cool (≈ 4 °C). There are two types of gelatin - Type A, derived from acid processed materials, primarily pork skin; and Type B, derived from alkaline or lime processed materials, primarily cattle or calf hides and ossein (cattle bones). Type A Gelatin is produced from fresh or frozen pork skin by washing with water and soaking in dilute acid.

Acidified skins are washed free of acid and soluble proteins. Treated porkskins are placed in extraction kettles and hydrolyzed with successive portions of hot water. The dilute solution is filtered and evaporated. Concentrated solutions are chilled to a gel which is then carefully dried with filtered and conditioned air in drying tunnels or in continuous dryers to a solid containing approximately ten percent moisture. The dried gelatin is then ground and tested for grade and quality. Calf skin or hide trimmings, sources of type B gelatins, are first washed and then treated with lime for one to three months. Limed skins are washed and neutralized with dilute acid. After this conditioning, the skin stock is transferred to kettles and heated with successive portions of hot water. A partial hydrolysis of the collagen occurs, resulting in extracts which are dilute solutions of gelatin. From this point on, the extracts of Type B gelatin are processed in a manner similar to Type A described above.

2.10.2 Bovine Serum Albumin (BSA)

Albumins are a group of acidic proteins which occur plentifully in the body fluids and tissues of mammals and in some plant seeds. Unlike globulins, albumins have comparatively low molecular weights, are soluble in water, are easily crystallized (can be precipitated by high concentrations of neutral salts such as ammonium sulfate), and contain an excess of acidic amino acids. Serum and plasma albumin is carbohydrate-free and comprises (55 to 62) % of the protein present. Albumin binds water, Ca^{2+}, Na^{+}, and K^{+}. Due to a hydrophobic cleft, albumin binds fatty acids which can alter the heat denaturation of the protein, bilirubin, hormones and drugs. BSA is a single polypeptide chain consisting of about 583 amino acid residues and no carbohydrates. At pH 5 to 7 it contains 17 intrachain disulfide bridges and 1 sulfhydryl group. The molecular weight of BSA are based on amino acid sequence. The solution stability of BSA is very good (especially if the solutions are stored as frozen aliquots). In fact, albumins are frequently used as stabilizers for other solubilized proteins (e.g., labile enzymes). However, albumin is readily coagulated by heat. When heated to 50 °C or above, albumin quite rapidly forms hydrophobic aggregates which do not revert to monomers upon cooling. At somewhat lower temperatures aggregation is also expected to occur, but at relatively slower rates. The main biological function of albumin is to regulate the colloidal osmotic pressure of

blood. Bovine albumins contain 16 % nitrogen and are often used as standards in protein calibration studies. Albumin is used to solubilize lipids, and is also used as a blocking agent in Western blots or ELISA applications. BSA is widely used because of its stability, its lack of effect in many biochemical reactions, and its low cost since large quantities of it can be readily purified from bovine blood, a byproduct of the cattle industry.

2.11 Immobilization of Biomolecules onto Polyester Surface

Due to the lack of chemical functionalities, it is usually difficult for polyesters to favor polymer–protein interaction, the covalent binding. The method of aminolysis between diamine and polyester matrix introduces functional amino groups, through which proteins such as gelatin, laminin, chitosan, Arg-Gly-Asp (RGD)-containing peptide, collagen etc., can be further introduced on the surface of polymeric scaffolds. There are many reviews on protein, peptide and/or growth factor immobilization onto the polymer matrices for use as scaffold for tissue engineering demonstrated follows.

Gao *et al.* (2003) modified polyurethane (PU) membranes by grafting polymerization of methacrylic acid (MAA) initiated by UV light and further covalently immobilization of gelatin or arginine-glycine-aspartic (RGD) peptide using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride as condensing agent. The results for endothelial cells cultured onto these membranes proved that the PU membrane modified with gelatin or RGD peptide had better cytocompatibililty than the control PU or the PMMA grafted PU membrane.

Ayhan *et al.* (2002) investigated nonwoven poly PL-lactide (PDLLA) matrices for tissue repair. PDLLA was synthesized by ring-opening polymerization of DL-lactide and nonwoven PDLLA matrices was carried out by glow-discharge treatment, followed by glutaraldehyde incorporation. Additionally, biological modification by immobilization of collagen and or fibronectin. Fibroblast-like, 3T3 and epithelial-like MDBK cells were selected as model cell. The modified matrices increased cell adhesion and proliferation compare to untreated PDLLA matrices.

Zhu *et al.* (2002a) covalently introduced amino groups onto a polycaprolactone (PCL) membrane by the reaction between 1,6-hexanediamine and the ester group of PCL, biocompatible macromolecules such as gelatin, chitosan or collagen were further immobilized on the aminolyzed PCL membrane via glutaraldehyde as a crosslink agent. The endothelial cell culture proved that the cytocompatibility of the aminolyzed PCL was improved due to the cell attachment and proliferation ratios were obviously improved.

Zhu *et al.* (2002b) modified polycaprolactone (PCL) membrane by grafting copolymerization of methacrylic acid (MAA) initiated under UV light and further covalently immobilized of gelatin onto the modified surface ,using condensing agent,1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride. The endothelial cell culture proved that the PCL membrane slightly modified with suitable amount of PMAA or gelatin had better cytocompatibility than control PCL or PCL membrane heavily modified with PMAA or gelatin.

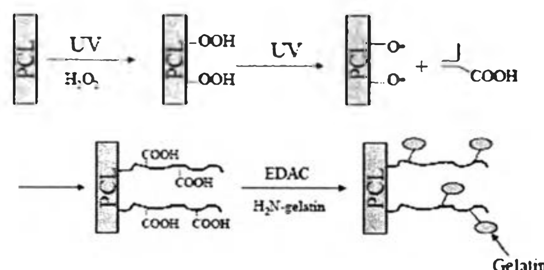

Figure 2.10 The schematic representation of photo-induced grafting with PMAA and further immobilization with gelatin on PCL membrane surface. (Zhu *et al.*, 2002b)

Zhu *et al.* (2004) developed several methods to enhance the cytocompatibility of cell and polymer. Poly-L-lactic acid (PLLA) was selected as matrix to compare the efficiency of the following method: 1) aminolysis (PLLA-NH_2) 2) collagen immobilization with glutaraldehyde (PLLA-GA-Col) 3) chondroitin sulfate (CS)/collagen layer-by-layer (LBL) assembly (PLLA-CS/Col) 4) photo-induced grafting copolymerization of hydrophilic methacrylic acid (MAA)

(PLLA-g-PMAA) and 5) futher immobilization of collagen with 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDAC) (PLLA-g-PMAA-col). Human umbilical endothelial cells (HUVECs) were culture onto these materials. The water contact angle measurement indicated that all the modification can improve the cytocompatibility of PLLA. All the collagen-modified PLLA also showed more positive cell response than those purely aminolyzed or PMAA grafted.

Cheng *et al.* (2004) prepared PCL film by using solvent casting and biaxial stretching technique. The PCL film was pretreated using Argon plasma, and then UV polymerized with acrylic acid (AAc). Collagen immobilization was then carried out. Results of water contact angle showed that the hydrophilicity of the surface has improved significantly after surface modification. Human dermal fibroblasts and myoblasts were seeded onto PCL films. The cell attachment and proliferation were improved remarkably on the modified surface. There was not only no increase of cell number on the unmodified PCL film surface, but also only a few cells were observed.

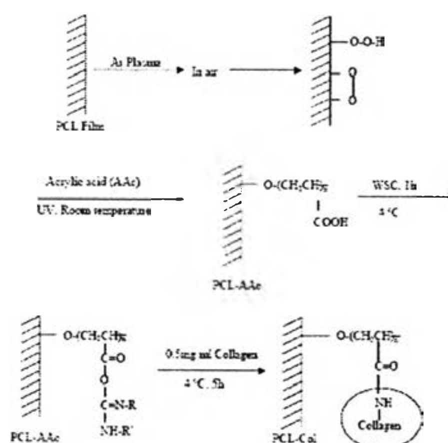

Figure 2.11 Schematic representation of the process of UV introduced AAc grafting and collagen immobilization (Cheng *et al.*, 2004).

Zhu *et al.* (2006) synthesized a biodegradable and flexible poly (L-lactide-co-caprolactone) (PLLC) copolymer. The surface modification of these materials via aminolysis by 1,6-hexanediamine to introduce free amino groups and linked covalently with fibronectin and collagen. Using glutaraldehyde as a coupling agent. Protein-bonded surface presented more hydrophilic and homogeneous cell culture

demonstrated. The modified PLLC is more favorable to epithelium tissue regeneration than the unmodified PLLC.

Santiago *et al.* (2006) prepared PCL disks with average thickness ~ (227.7 ± 16.3) μm (determined by SEM) and modified their surface with 1,6 hexanediamine in order to promote the attachment and proliferation of adipose-derived stem cell (ASCs; as a source of adult stem cells for tissue engineering application: bone, cartilage). Peptide sequences derived from ECM protein laminin were each covalently attached to an aminated polymer surface using carbodiimide. A high number of cell observed in the treated PCL disk in comparison with untreated PCL. This study indicated that immobilization of peptide sequences has potential in promoting the attachment ASCs to biocompatible scaffold.

Edlund *et al.* (2008) presented surface modification pathway of poly(L-lactide) and poly(ε-caprolactone) (PCL) scaffold in a three-step coupling procedure. In this case, a growth factor polymers scaffold was heparin covalently bonded to the surface and an osteoinductive growth factor immobilized in the heparin layer. The result showed that the biocompatibility of functionalized surfaces much improved cell attachment and proliferation of mesenchymal stem cells (MSCs) as compared to a physical coating of BML-2 to a substrate.

Shen *et al.* (2009) investigated the immobilization of rhBMP-2 on poly acetone-type polymer scaffolds via plasma treatment under different atmospheres (oxygen, ammonia and carbon dioxide) to introduce functional groups on the surface of PLGA matrix. The effect of various plasma-treated PLGA film on binding rhBMP-2 was studied and compared. The result showed that the binding ability of the oxygen plasma-treated PLGA to rhBMP-2 played main roles in improving the binding ability. Mouse OCT-1 osteoblast-like cell as a model cell was cultivated on the rhBMP-2 bound oxygen-treated PLGA. The immobilized rhBMP-2 had stimulated differentiation of OCT-1 cell and accelerated process of mineralization of OCT-1 in the scaffold.

Savarino *et al.* (2007) have investigated the ability of cellular construction of poly-ε-caprolactone (PCL) porous scaffold, seeded with autologous rabbit bone marrow stromal cells (BMSCs) and bone morphogenetic protein 4-expressing

BMSCs. The result scarce bone formation and scarce bone resorption in PCL without cell. In constrast, PCL seeded with growth factor expressed cell stimulated new tissue formation. PCL alone did only induce a minor foreign body reaction while the potential action of BMSCs and BMP on the increased recruitment of inflammatory cell both around and into PCL scaffold remains to be clarified.

Mattanavee *et al.*(2009) prepared PCL in the form of electrospun fibrous scaffolds, first modified with 1,6-hexamethylenediamine to introduce amino groups on their surface, and then immobilized various biomolecules, i.e., collagen, chitosan, and Gly-Arg-Gly-Asp-Ser (GRGDS) peptide on their surface, with *N,N'*-disuccinimidylcarbonate being used as the coupling agent. Three different cell lines, e.g., mouse fibroblasts (L929), human epidermal keratinocytes (HEK001), and mouse calvaria-derived preosteoblastic cells (MC3T3-E1) were cultured to evaluate the potential for use of these PCL fibrous scaffolds. the result demonstrate that type I collagen- immobilized PCL fibrous scaffolds showed the greatest ability to support the attachment and proliferation of all of the investigated cell types, followed by those that had been immobilized with GRGDS peptide and neat PCL scaffold.

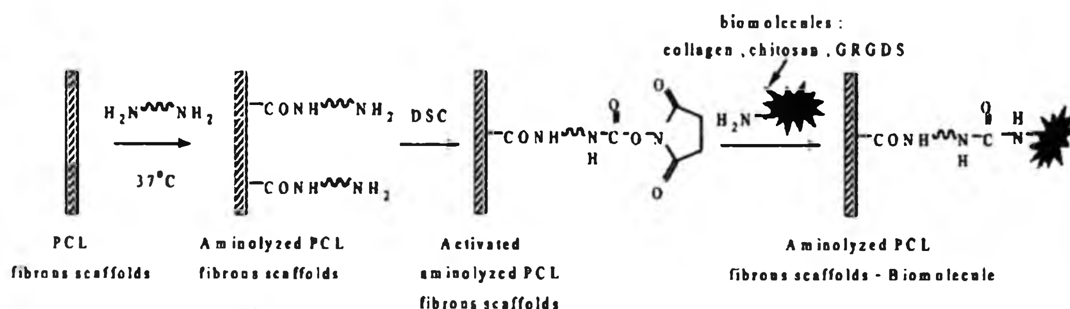

Figure 2.12 Chemical pathway for the immobilization of different biomolecules, such as collagen, chitosan, or GRGDS peptide (i.e., H-Gly-Arg-Cly-Asp-Ser-OH), on the surface of the electrospun PCL fibrous scaffolds (Mattanavee *et al.*, 2009).

The present purpose focuses on improving surface hydrophilicity of electrospun PCL fiber mats by aminolysis with 1,6-hexamethylenediamine to introduce free amino groups onto a ester-containing polymer surface. Many type of proteins; i.e.gelatin type-A, gelatin type-B, bovine serum albumin and crude bone

protein were further immobilized using *N, N'*-disuccinimidyl carbonate (DSC) as a coupling agent. Direct cytotoxicity test was firstly use to evaluate the toxicity of all proteins (used in this study) to cells. Indirect cytotoxicity evaluation of the aminolyzed, activated, protein-immobilized PCL electrospun fibrous scaffold based on pre-osteoblasts (MC3T3-E1) was subsequently investigated. Morphological appearance of pre-osteoblasts (MC3T3-E1) cultured on these modified electrospun fibrous scaffolds was observed. The potential use of surface-modified PCL electrospun fibrous mats as bone scaffolds was further evaluated *in vitro* with MC3T3-E1 in terms of attachment, proliferation, and differentiation of the cells that were cultured on the scaffolds. For differentiation study, the ALP activity was used as an early marker of differentiation and mineralization experiment was used to determine the quanlity of mineral substances (calcium and phosphate) to which MC3T3-E1 differentiated and changed. The results were compared with those of TCPS.