CHAPTER II
LITERATURE REVIEW

2.1 Bone Tissue Engineering

Bone Tissue Engineering is an emerging interdisciplinary field that seeks to address the needs by applying the principles of biology and engineering to the development of viable substitutes that restore and maintain the function of human bone tissues. There are many approaches to bone tissue engineering, but all involve in the following key ingredients: harvested cells, recombinant signaling molecules, and three-dimensional (3D) matrices (Caenegie Mellon University). Tissue engineering for bone typically involves coupling osteogenic cells and/or osteoinductive growth factors with osteoconductive scaffold. In terms of osteoinductive growth factors, most research has focused on the use of the bone morphogenic protein (BMPs) and, in particular, BMP-2 (Chu *et al.*, 2007).

The structural cues involve the interaction of bone cells (osteoblasts, osteoclasts, osteocytes) exist in a symbiotic relationship with extracellular matrix (ECM). The ECM is a complex structural entity surrounding and supporting cells that part of our body which gives it form and shape. Placing the cells and growth factors in synthetic scaffolds that act as temporary ECMs (Lee, Kuen Yong *et al.*, 2007), (Caenegie Mellon University). The ECM is often referred to as the connective tissue. The ECM is composed of 2 major classes of substances:

1. Organic substance contains 25% of ECM. It is composed of collageneous proteins (90% - collagen type I) collagens are the most abundant proteins found in the animal kingdom. It is the major protein comprising the ECM and noncollageneous proteins include (1) glycoproteins such as osteonectin (binds Ca^{2+} and collagen; nucleates hydroxyapatite) and osteopontin (constituent of cement line; involved in bone remodeling) (Salgado, A.J *et al.*, 2004). (2) Osteocalcin are embedded in the extracellular matrix and may have important signaling functions (bone morphogeneic proteins, growth factors, cytokines, adhesion molecules) or play a role during the mineralization process (osteopontin, osteonectin, and matrix-

glaprotein) (Sommerfeldt, D.W. *et al.*, 2001), (3) Proteoglycans - 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 and store within the ECM, lipids (ground substance) and (4) γ-carboxyglutamic acid containing proteins (vitamin K dependent).

2. Nonorganic 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 20–80 nm in length and 2–5 nm thick. Because 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. *et al.*, 1992).

And the other component is water (5 %).

2.2 Growth Factor in Bone Regeneration

Bone formation is a complex process that involves a large number of hormones, cytokines and growth factor (Carano, R.A. *et al.*, 2003). Due to advancement, tissue engineering strategies utilize combination of cells, biodegradable scaffolds, and bioactive molecules i.e. growth factors to run through natural processes of tissue regeneration and development. Growth factors involve bone regeneration and other processes of bone are osteoinductive growth factors, such as bone morphogenetic protein (BMP), transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), insulin-like growth factors (IGF) and fibroblast growth factors (FGFs) have been investigated to induce bone regeneration at the bone defect, especially BMPs (H.Li *et al.*, 2001).

2.3 Growth Factor Carrier Materials

One approach in tissue engineering includes delivery of growth factors from optimally designed biodegradable carriers to stimulate cellular adhesion, proliferation, and differentiation in order to promoting bone regeneration. 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, the role of 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 concern 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.

To guide *in vitro* or *in vivo* tissue regeneration, it is necessary to obtain appropriate delivery carrier material (Almirall, A. *et al.*, 2004) which satisfy all the goals required: (1) the delivery carrier should be biocompatible, noncytotoxic, nonimmunogenic potentiality, osteoconductivity, hydrophobicity, protect proteins from non specific lysis and serve to prolong exposure of the proteins to cells for increasing the duration of the proteins interaction with other growth and differentiative (Lee, K.Y., 2007), (2) implanted carriers should be degraded by enzymes and/or circulating biological fluid, (3) the carrier should have high interconnected macroporosity to allow cellular infiltration and ingrowth (Almirall, A. *et al.*, 2004), (4) the design of bone tissue engineering scaffolds should maintain appropriate mechanical stability and sufficient mechanical strength.

2.3.1 Organic Matrices

Organic matrices comprise two groups: Natural polymer and Synthetic polymers.

2.3.1.1 Natural Polymer

Natural polymer such as collagen, fibrin, alginate, silk, hyaluronic acid, and chitosan. Advantages are biocompatible, degradable and solubilized in physiological fluid. Disadvantages are immunogenicity, difficulty in processing, and a potential risk of transmitting animal-originated pathogens (Lee, Soo-Hong *et al.*, 2007).

2.3.1.2 Synthesic Polymer

Synthesic polymer such as PLA, PLLA, PGA, PLGA, PCL, PPF, PEG etc. Polylactone-type biodegradable polymers, such as PLLA, (PGA) and (PLGA) are extensively studied as scaffold materials for tissue engineering (Sachlos, E. *et al.*, 2003). Advantages are excellent chemical and mechanical properties, associated with their processibility and flexibility to tailor to have appropriate chemical and mechanical properties. Disadvantages are chronic inflammatory response, potential localized pH decrease due to relative acidity of hydrolytically degraded by product, retarded clearance rate, and limited biological function (Soo-Hong Lee *et al.*, 2007).

2.3.2 Inorganic Matrices

Inorganic matrices such as BCP (biphasic calcium phosphate) is a bioceramic material which consists of a mixture of hydroxyapatite (HAp) and β-tricalcium- phosphate (β-TCP), HAp (hydroxyapatite), TCP (tricalcium phosphate), ACP (amorphous calcium phosphate), CA (carbonated apatite), CDHA (Calcium-deficient), bioactive glasses, other inorganic matrices. HAp and β-TCP have been widely applied as bone substitutes for artificial bone grafts. Their chemical composition close to the mineral phase of bone is an origin of their excellent biocompatility to tissue bone. Both HAp and β-TCP are strong structure, immunologically inert, biocompatibility, and osteoconductive, but they lack osteoinductive (Sopyan, I. *et al.*, 2007), brittleness and low strength limited (Ono, I. *et al.*, 1998).

2.3.3 Composite Matrics

Composite materials of organic/inorganic origin are studied extensively because they can combine the tailored degradability and high release efficiencies of the polymer with the osteoconductiveity and delayed/sustained release characteristics of the ceramic material. Mechanical and physical properties of the polymer can compromise the brittleness of the ceramic material or give better handling properties to calcium phosphate cement (Habraken, W.J.E.M *et al.*, 2007).

2.4 Growth factor delivery strategies.

Growth factor delivery through scaffolds could be achieved either through direct incorporation of growth factor or encapsulation of growth factor secreting cells into scaffold. Alternatively plasmid DNA of growth factors can be encapsulated within the scaffolds, leading to the production of growth factors within cells. Scaffolds have been utilized in the design of tissue engineered constructs in delivering several growth factors for the correction of a wide range of medical conditions. A variety of scaffolds have been used to deliver growth factors, generally form either hydrogel or solid scaffolds. The various techniques currently employed in the incorporation of growth factors within scaffolds and can be broadly divided into two methods — attachment of the growth factors into the scaffold and the physical entrapment of growth factor within the scaffold. Systems for the controlled release of peptides and proteins involve encapsulation or entrapment of proteins in biocompatible polymeric devices (Sokolsky-Papkov, M. *et al.*, 2007).

2.4.1 Attachment of Growth Factors to Scaffold

These methods generally involve the attachment of growth factors onto preformed scaffolds. These methods could involve the immobilization of the growth factor onto the surface of the scaffold through chemical crosslinking, or the adsorption of the growth factor into the scaffold such as dip coating (Arm, D.M. *et al.*, 1996), immerse (Ono, I. *et al.*, 1996), mixing (Murata, M. *et al.*, 1998), impregnation (Alam, Md. Imranul *et al.*, 2001), pipetting (Sumner, D.R. *et al.*, 2001), dope (Schnettler, R. *et al.*, 2003), and dropping (Takahashi, Y. *et al.*, 2005). HAp is very important to regulate the HAp resorption in the body and to control the release of growth factors at the optimal time and amount (Matsumoto, T. *et al.*, 2004).

2.4.2 Entrapment of Growth Factors Within Scaffold

In addition to the attachment of growth factors to polymeric scaffolds, growth factors can be directly incorporated within scaffolds during the fabrication process. These methods generally involve the mixing of the polymers with the growth factor before processing to form the scaffold. The main challenge of this set

of methods is to ensure that the processing conditions do not significantly denature the incorporated growth factors while still able to secrete the growth factor in a sustained manner (Sokolsky-Papkov, M. *et al.*, 2007).

2.4.2.1 Hydrogel Scaffolds

Three types of hydrogel-based protein delivery systems have been studied (Fig. 2.1). Physically entangled polymer systems slowly dissolve and release the protein simultaneously with polymer dissolution (Fig. 2.1i). Chemically crosslinked polymer gels degrade due to hydrolysis or enzymatic digestion (Fig. 2.1ii); in these delivery systems, protein is released at a rate that is dependent on the rate of polymer degradation. Hydrogels that swell after contact with water permit diffusion of macromolecules throughout the entire matrix, so that agents are released through a porous structure that expands during swelling (Fig. 2.1iii). The size of the pore located within the network, which is related to the extent of crosslinking and the degree of swelling, determines the protein release rate. The rate of protein release from hydrogel networks can be modified by varying the degree of physical entanglement within the gel, by altering the number of chemical crosslinks between the polymer or by altering the interactions between the polymer matrix and the molecule of interest. For example, polymer–protein interaction can be modified by the use of ionizable groups on the polymer network, so that oppositely charged molecules are stabilized by the matrix and like-charged molecules are excluded from the polymer matrix (Baldwin, S. *et al.*, 1998). In corporation of protein in poorly crystalline carbonate apatite hydrogel was prepared by precipitation of saturated solution of calcium and phosphate in air or under N_2 atmosphere. The quantity of adsorbed protein should depend on the specific surface area of apatite hydrogel. The decrease in weight of apatite cake prepared from hydrogel formed in air was larger than that from under N_2 (Yokogawa, Y. *et al.*, 2006).

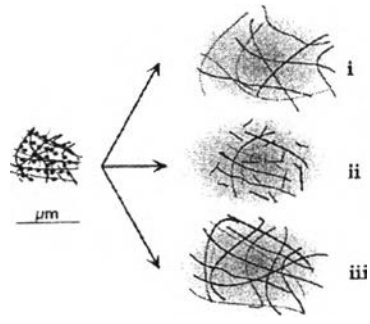

Figure 2.1 Release from physically entangled (i), degradable (ii), and swelling (iii)

2.4.2.2 Porous Solid Scaffolds

Formation of growth factor loaded porous solid scaffolds can be achieved through several techniques. The main techniques involve either one step direct formation of growth factor loaded scaffolds or formation of growth factor loaded microspheres which can be then be fabricated to form scaffolds.

Scaffold fabrication by solvent casting / particulate leaching. This technique involves the pouring of the polymer solution into a bed of salt particles with defined size. Precipitation of the polymer by evaporation of the polymer solvent under vacuum, followed by leaching of the salt particles in distilled water leads to the formation of a highly porous scaffold with well defined pores. This process of scaffold production is a popular technique and has been used in the production of angiogenic factors for the induction of angiogenesis.

Supercritical fluid. The benefits of using these process methodologies are due to the ability to form growth factor loaded scaffolds in conditions that are favourable to growth factors, avoiding the need for using organic solvents and harsh process conditions. CO_2 is the most common candidate for use as a SCF due to its low toxicity, ease of use and cost (Yokogawa, Y. *et al.*, 2006).

2.4.2.3 Electrospun Scaffolds

The electrospinning process produces fibers with nanoscale diameters through the action of a high electric field. This process involves the subjecting of a polymer solution in a capillary to an electric field generated by high voltage (Yokogawa, Y. *et al.*, 2006).

2.4.2.4 Microspheres Based Scaffolds

Microsphere can be produced in a wide range of sizes (1-100 nm for nanospheres, and 1-100 μm for microsphere) (Baldwin, S. *et al.*, 1998). Growth factor loaded microsphere can be formed either through chemical cross-linking or by solvent extraction. Growth factor released from these microspheres was shown to increase the proliferation of human umbilical vein endothelial cells in culture. Growth factor loaded microspheres can be used for tissue engineering applications either by direct fusing of microspheres directly to form scaffolds, or combination with other scaffold forming materials to form composite scaffolds. (Sokolsky-Papkov, M. *et al.*, 2007). One of the main advantages of microsphere formulation is to provide stability for labile compounds that are rapidly degraded or cleared out in vivo. Drug release from the microsphere occurs by several mechanisms including diffusion, polymer degradation, hydrolysis or erosion. (Degim, I. *et al.*, 2007). The main product methods are depicted in Fig 2.2

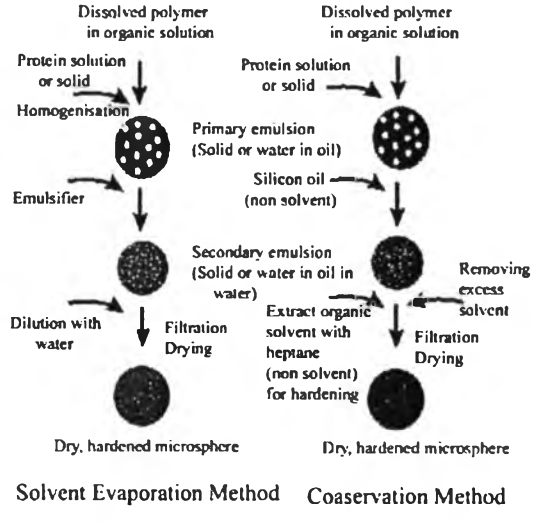

Figure 2.2 Production methods of polymer microsphere.

2.5 Release of protein

2.5.1 Factors Affecting Protein Release Rate

Controlled release is an attainable and desirable characteristic for protein delivery systems. The factors affecting the protein release rate revolve around the structure of the matrix where the protein is contained and the chemical properties associated with both the polymer and the protein. A protein encapsulated in a slowly degrading matrix provides the opportunity for slower release effects, but polymer degradation is degradation is not the only mechanism for release of proteins. The protein release is also diffusion controlled as the drug can travel through the pores. In some cases, proteins containing nucleophilic groups can cause increased chain scission of the polymer matrix, which also increases the rate of protein expulsion. Polymer molecular weight, drug distribution, polymer blending, crystallinity, and other factors are important manipulating release profiles. The most desirable release profile would show a constant release profiles are more complicated and often contain two main expulsion processes: the first being an initial burst of expelled proteins from the surface; the second, a usually more constant stage with release rates depent on diffusion and degradation. The release profiles are also dependent on the size of the particles; the rate of protein release was found to decrease with increasing the particle size (Freiberg *et al.*, 2004).

Controlled release dosage from of peptides and proteins have been prepared as implants. Implantable systems have many advantages, including prolonged effect and targeting (Degim, I. *et al.*, 2007).

2.5.2 Protein Release from Microsphere Pores of HAp

Rate of protein release is through to depend mainly on the rate of polymer degradation and protein diffusion through the microsphere pores (Ho, M.L. *et al.*, 2008) release studies as a function of pH (IJntema, K *et al.*, 1994). The release of BSA from BSA-load HAp microcarriers is expected to take place via two processes : (a) BSA desorption and (b) HAp dissolution.

2.5.3 Protein release from porous emulsion-coated scaffolds

The difference in release profile (constant vs. decreasing release rate) is most likely related to the geometry and dimensions of the matrices. The increase in

porosity may account for the faster release. It is well known that the emulsification procedure used to prepare protein-loaded coatings may cause protein aggregation and incomplete release, as well as a decreased enzymatic activity. The release of a model protein (lysozyme) from the coated scaffolds could effectively be tailored from 3 days to more than 2 months, by varying process parameters such as emulsion copolymer composition or water/polymer ratio (Sohier, J. *et al.*, 2003). The dissolution of HAp particles was also evaluated in relation to the elapsed time with different pH solutions in order to estimate the relation between the protein release and the HAp dissolution (Matsumoto, T. *et al.*, 2004).

2.6 Biomaterials of Choice

2.6.1 Hydroxyapatite (HAp)

HAp is a naturally occurring form of calcium apatite with the formula $Ca_{10}(PO_4)_6(OH)_2$. HAp is the hydroxyl endmember of the complex apatite group. The OH^- ion can be replaced by fluoride (F^-), chloride (Cl^-) or carbonate (CO_3^{2-}). It crystallizes in the hexagonal crystal system. It has a specific gravity of 3.08 and is 5 on the Mohs hardness scale. Pure HAp powder is white. Naturally occurring apatites can however also have brown, yellow or green colorations, comparable to the discolorations of dental fluorosis (http://www.wikipedia.org). HAp is chemically similar to the mineral component of bones and hard tissues in mammals. It is one of few materials that are classed as bioactive, meaning that it will support bone ingrowth and osseointegration when used in orthopaedic, dental and maxillofacial applications (http://www.azom.com).

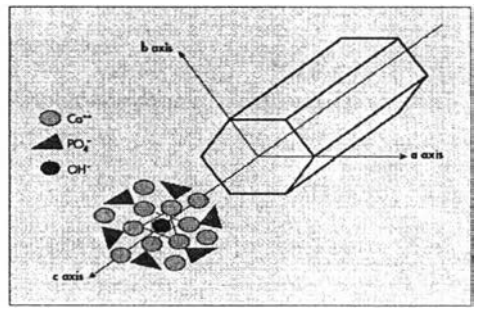

Figure 2.3 Schematic illustration of the unit cell from a crystal of hydroxyapatite.

HAp of bioactive ceramics have been extensively investigated as the cell scaffold for bone tissue engineering because it has good compatibility (Takahashi, Y. *et al.*, 2005), bioaffinity, mechanical properties, stimulates osteoconductivity, and is slowly replaced by the host bone after implantation. For sintered hydroxyapatite to fit into a bone cavity, the surgeon needs to machine the graft to the desired shape or carve the surgical site around the implant. This leads to increases in bone loss, trauma and surgical time (Xu Hockin, H.K. *et al.*, 2008). Disadvantages are poor biodegradability, brittle nature, difficult to freely change the shape (Takahashi, Y. *et al.*, 2005), poorly resorbed and hampers rapid bone turnover in the affected area. Therefore, using a combination of HAp and TCP ceramics (HAp/TCP), bone replacement can be enhanced since TCP displays better bioresorption. Generally speaking dense hydroxyapatite does not have the mechanical strength to enable it to succeed in long term load bearing applications (Imranul, A. *et al.*, 2001).

2.6.1.1 Hydroxyapatite Production Techniques

HAp has been synthesized by many methods such as ultrasonic precipitation (Cao, Li-Yun *et al.*, 2005), precipitation (Monmaturapoj., 2008), neutralization (Smiciklas *et al.*, 2005), emulsion (Ho, M.L. *et al.*, 2008), microemulsion (Koumoulidis *et al.*, 2003), inverse microemulsion (Lim, G.K. *et al.*, 1996), hydrothermal (Zhang, X. *et al.*, 2007), sonochemical (Kim *et al.*, 2001), hydrogel (Yokogawa *et al.*, 2006), and Hydrolysis method (Shih, Wei-Jen *et al.*, 2004). In these methods were usually used to prepare HAp powders because it is easy to operate and need not any expensive equipment (Cao, Li-Yun *et al.*, 2005). Variation in these routes leads to some differences in morphology, crystallographic structures and density (Afshar, A. *et al.*, 2003). Researchers have been studied the influence of various parameters on synthesizing HAp such as precursor reagent, impurity contents, concentration, (Boonsongrit, Y. *et al.*, 2008), pH, temperature (Li, Y. *et al.*, 2008), (Matsumoto, T. *et al.*, 2004), atmosphere (Yokogawa, Y. *et al.*, 2006), and surfactant addition (Li, Y. *et al.*, 2008). The following starting materials are used: calcium carbonate, calcium hydroxide, calcium oxide, calcium nitrate, organoacidic calcium salts, calcium hydrogen phosphate and others (Suwa, Y. *et al.*, 1993). Higher temperature produced HAp with larger proportion of crystalline phase,

morphology of the HAp samples became more well-defined, and tighter packing of HAp (Li, Y. *et al.*, 2008). The crystallinity of HAp depends on its synthesized temperature in such a way that HAp synthesized at low temperature has low crystallinity. The solubility of HAp also depended on its synthesized temperature showed high solubility (Matsumoto, T. et al., 2004). Content of the coarser granules increases generally with a decrease in the stirring rate and the oil temperature. The temperature effect is due to both the increased viscosity and surface tension of the oil). An increase in sintering temperature decreases the open content (Komlev, V. *et al.*, 2002). Porous scaffolds are at the heart of bone tissue engineering approaches. The features of scaffolds are important for regeneration of the bone. Using traditional scaffold manufacturing technologies, such as fiber bonding, solvent casting, particulate leaching, gas saturation and membrane lamination. It is difficult to make customized scaffolds with specially designed functional gradient material, for instant different porosity and surface structures. Recently natural hydroxyapatite bioceramics have been extracted by normal calcinations of some biowasts e.g. bovine bones (Barakat, N. *et al.*, 2009) and eggshell (Acevedo-Davila, J.L. *et al.*, 2007).

Scaffolds are central components of many tissue engineering strategies because they provide an architectural context in which extracellular matrix, cell–cell and growth factor interactions combine to generate regenerative niches. There is a significant challenge in the design and manufacture of scaffolds that possess both a highly porous structure and the ability to control the release kinetics degradation of growth factors over the period of tissue regeneration and protect protein from enzymatic. The criteria for choosing materials to act as the foundation for a scaffold are challenging. The materials used must be safe, not cause excessive immune responses, possess acceptable biocompatibility, be non-toxic and erodable. On the other hand, the materials must possess appropriate mechanical properties and be suitable for manufacturing techniques that generate high surface area porous structures. Porous ceramics show two main advantages compared to dense ceramics. One of them deals with their high surface area, which in chemical terms involves higher reactivity and consequently faster bone regeneration. In fact, the surface area of these materials can be turned depending on the final properties desired. The second advantage consists on their ordered porosity framework that allows

homogeneously loading and releasing of drugs. On the other hand, they present the disadvantage of being even more fragile than dense ceramics (M.Vallet *et al.*, 2008). Different types of CaP-ceramics are available, though they can be classified as either hydroxyapatite (HAp), beta-tricalcium phosphate (β-TCP), biphasic calcium phosphate (ACP), carbonated apatite (CA) or calcium deficient HAp (CDHA). Calcium phosphate cements are of interest for bone tissue engineering purposes. In general there are two types of CaP cement: apatite cements that set into HAp, CA or CDHA and brushite cements that have DCPD (brushite) as end product (Habraken, W.J.E.M. *et al.*, 2007).

Table 2.1 Abbreviations of CaP-compounds with corresponding chemical formula and Ca/P ratio (Habraken, W.J.E.M. *et al.*, 2007)

Abbreviation	Name	Formula	Ca/P-ratio
ACP	Amorphous calcium phosphate	–	$1.25<x<1.55$
BCP	Biphasic calcium phosphate	$Ca_3(PO_4)_2+Ca_{10}(PO_4)_6(OH)_2$	$1.50<x<1.67$
CA	Carbonated apatite, dahlite	$Ca_5(PO_4,CO_3)_3$	1.67
CDHA	Calcium deficient hydroxyapatite	$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$	$1.50<x<1.67$
DCPA	Dicalcium phosphate anhydrous, Monetite	$CaHPO_4$	1.00
DCPD	Dicalcium phosphate dihydrate, Brushite	$CaHPO_4{\cdot}H_2O$	1.00
HA	[illegible]	[illegible]	[illegible]
MCPM	Monocalcium phosphate monohydrate	$Ca(H_2PO_4)_2{\cdot}H_2O$	0.50
OCP	Octacalcium phosphate	$Ca_8H_2(PO_4)_6{\cdot}5H_2O$	1.33
pHA	Precipitated hydroxyapatite	$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$	$1.50<x<1.67$
α-TCP	α-Tricalcium phosphate, Whitlockite	$\alpha\text{-}Ca_3(PO_4)_2$	1.50
β-TCP	β-Tricalcium phosphate, Whitlockite	$\beta\text{-}Ca_3(PO_4)_2$	1.50
TTCP	Tetracalcium phosphate, Hilgenstockite	$CaO{\cdot}Ca_3(PO_4)_2$	2.00

2.6.2 Ovalbumin

Ovalbumin is a major protein found in the white of hens'eggs (60-65% of the total protein), where it acts as a storage protein providing nutrients for the developing chick embryo (Hemingway, C.A. *et al.*, 2008). It is the predominant protein in egg white, comprising approximately 54% of the total protein. It is a complex protein, consisting of a single polypeptide chain of 385 amino acid residues, with a maximum of 2 phosphate residues per mole, and an oligosaccharide side chain composed of mannose and glucosamine residues. The relative molecular mass is approximately 45 kDa. Ovalbumin is very similar in amino acid content to bovine serum albumin (BSA) (see in Table 2.2) and can be an excellent substitute for BSA. An important advantage of ovalbumin is the elimination of the risk of disease

associated with bovine and human derived albumins, because ovalbumin originates from a safer starting material than the other albumins and is processed to a pure form under more sanitary conditions. In addition, ovalbumin may be used in cell culture systems and in the diagnostic industry to stabilize enzymes and hormones that would otherwise lose their functional integrity. It is also ideal as a reference protein for both immunological and biochemical studies, where it serves as an effective carrier and as a stabilizer protein. Mesoporous polycrystals of HAp-CPP are synthesized via a biomineralizing route using ovalbumin. (http://www.fordras.com/ovalbumin).

Table 2.2 Comparison of amino acid between albumin (g/100g of protein)

AMINO ACIDS :	OVALBUMIN	LACTALBUMIN	BSA
LYSINE :	6.6	11.5	12.8
HISTIDINE :	2.3	2.9	4.0
ARGININE :	5.9	1.2	5.9
ASPARTIC ACID :	9.4	18.7	10.9
THREONINE :	4.5	5.5	5.8
SERINE :	8.1	4.8	4.2
GLUTAMIC ACID :	16.1	12.9	16.5
PROLINE :	3.6	1.5	4.8
GLYCINE :	3.2	3.2	1.8
ALANINE :	5.8	2.1	6.3
HALF CYSTINE :	2.4	6.4	6.5
VALINE :	7.1	4.7	5.9
METHIONINE :	4.9	1.4	0.8
ISOLEUCINE :	7.0	6.8	2.6
LEUCINE :	10.1	11.5	12.3
TYROSINE :	3.9	5.4	5.1
PHENYLALANINE :	5.4	4.5	6.6
TRYPTOPHAN :	1.2	5.3	0.6

2.6.3 Gelatin

Gelatin is a heterogeneous mixture of single or multiple stranded polypeptides (and their oligomers) each of which contains about 300-4000 amino acids. Gelatin is typically derived from the triple helix type I collagen from skin and bones. Gelatin can be produced by two different processes (Figure 2.4) depending on the method in which collagen are pretreated prior to be extracted. The alkaline process (or liming) targets the amide groups of asparagines and glutamine residues, hydrolyses them into carboxyl groups, and eventually converts them to be aspartate and glutamate. The alkaline treated gelatin possesses more of carboxyl groups (so call acidic gelatin or type B), thus charges negatively and presents lower isoelectric

point (*pI*) at about 3-5. On the contrary, the acidic process hardly reacts to the amide groups of collagen. As a result, acid treated gelatin (so call basic gelatin or type A) posses *pI* similar to that of collagen at about 7-9.

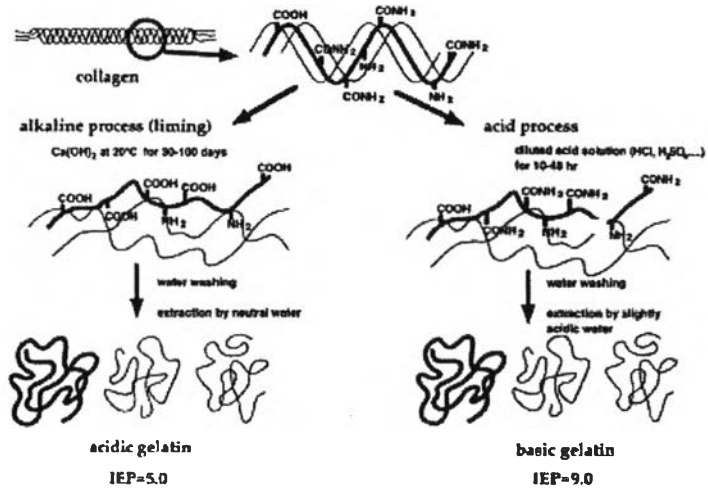

Figure 2.4 Process of gelatin production (Tabata and Ikada, 1998)

Gelatin structurally contains many glycine, proline and 4-hydroxyproline residues. A typical structure is -Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro- in which glycine is almost 1 in 3 residues, and arranged at every third residue (Figure 2.5).

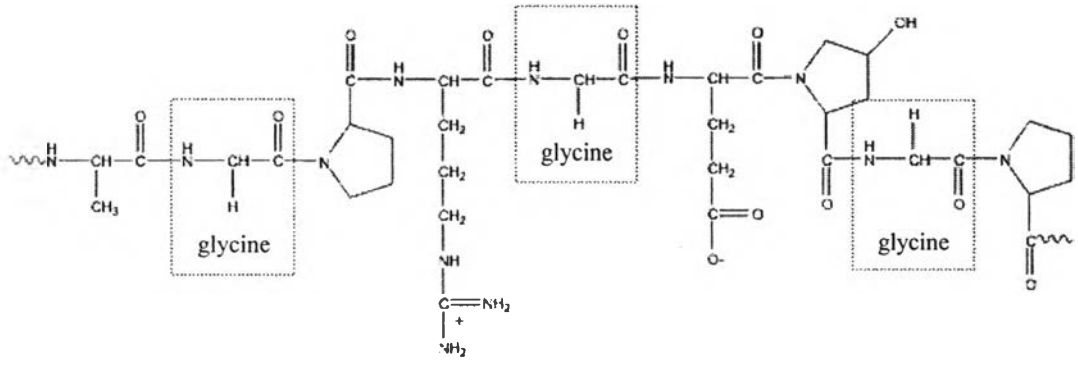

Figure 2.5 Chemical structure of gelatin (Chaplin, *et al.*, 2007)

Gelatin, a natural polymer, is commonly used in pharmaceutical and medical application due to the biocompatibility and biodegradability. The diversity

of gelatin's pI can be selectively used to from complex with the oppositely charged molecule like proteins, to be the polyion complexation which is quite stable and can function as a matrix for sustained release of therapeutic proteins.

2.6.4 Bovine Serum Albumin

Bovine serum albumin is a large globular protein (66,000 Da) with a good essential amino profile. BSA is an acidic protein with isoelectric point of 4.2-4.8 is well known to mainly adsorb on the Ca-sites on HAp by ionic interaction between carboxyl acid groups on BSA and the positively charged Ca-sites. (Boonsongrit, Y. *et al.*, 2008).

2.6.5 Crude Bone Protein

Bone is physically a hard tissue abundant with mineralized extracellular matrix which makes up about 90% of the total weight of compact bone. Bone extracellular matrix comprises 60% of the microcrystalline calcium phosphate resembling hydroxyapatite, 27% of fibrillar type I collagen and 3% of the minor collagen types and other bone proteins including osteocalcin, osteonectin, phosphoproteins, sialoproteins and glycoproteins, as well as proteoglycans, glycoaminoglycans, and lipids (Hauschka *et al.*, 1986).

Bone extracellular matrix apparently is a depository of bioactive proteins and peptides. In healthy tissue, bone extracellular matrix controls the presentation and distribution of growth factors accurately for any specific condition (Hauschka *et al.*, 1986). Bioactivities of the existent growth factors in bone extracellular matrix retain even in the demineralized condition. In the mid-60s, Urist (Urist *et al* 1965) demonstrated the induction of new ectopic bone formation from a decalcified bone matrix and proposed the existence of osteoinductive molecules in the matrix that direct differentiation of precursor cells into bone forming osteoblasts. The study of Somerman et al. in 1983 (Somerman *et al.*, 1983) confirmed the proposition. They reported that demineratized bone matrix extracted with 0.05 M Tris HCl, pH 7.2 containing 4 M guanidine HCl contained all the information necessary for bone formation. The extracts illustrated chemotactic activity to osteoblast-like cells and itself was heat-labile and sensitive to trypsin, which therefore is a protein-like material. The deminearalized bone powders induced new bone formation when implanted subcutaneously.

Bone extracts form the demineralized bovine bone powder was investigated the growth factors in a quantitative fashion by Hauschka et al. in 1986 (Hauschka *et al.*, 1986). The result illustrated abundant growth factor activity as high as 570 GFU/g of dry bone (200 pg protein/GFU) in the extracts obtained by 0.5 M EDTA, 0.05 M Tris-HCl, pH 7.5 extractions. The isolated crude bone extracts and growth factors significantly stimulated mitogenic activity on osteoblast of the newborn rat calvaria while depressing alkaline phosphatase specific activity by 2-3 folds.

Extracts from bone contain a lot of factors for chemotaxis, attachment, proliferation and differentiation of bone cells. In 2000, Hou L.T. studied the effects of bone extracts on behaviors of human periodontal fibroblasts *in vitro* and revealed that the periodontal fibroblasts cultured with bone extracts possess osteoblastic characteristics by presenting genetic expression of alkaline phosphatase, osteocalcin, osteonectin, sialoproteins and bone morphogenetic protein-7 and were also capable of forming mineralized foci *in vitro*. The results corresponded to those of the previous studies which also demonstrated that extracts of bone matrix regulate osteoblast function (Syftestad and Caplan, 1984; Cho *et al.*, 1992).

Crude bone extracts, therefore, evidently encompasses enormous active proteins and growth factors which facilitates new bone formation. Extraction of the crude bone protein with the intricate procedures seems not to obliterate bioactivities of those factors. These presumptions initiate the plan to exploit crude proteins extracted from demineralized bone in regenerating bone tissue in this thesis.

2.6.6 Polycaprolactone (PCL)

PCL is a biodegradable polyester with a low melting point of around 60°C and a glass transition temperature of about -60°C. PCL can be prepared by ring opening polymerization of ε-caprolactone using a catalyst such as stannous octanoate. It is degraded by hydrolysis of its ester linkages in physiological conditions (such as in the human body) therefore it 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. The structure of PCL is shown in Figure 2.6.

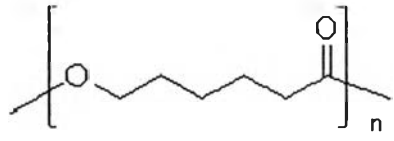

Figure 2.6 The structure of polycaprolactone.