CHAPTER II
LITERATURE REVIEW

2.1 Tissue Engineering

2.1.1 Fundamentals of Tissue Engineering

Tissue engineering is the new approach to overcome the limitations of the existing therapies for the treatment of malfunctioning or lost organs. 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 function. Generally, biocompatible and biodegradable polymer is used in tissue engineering to allow the growth of the tissue surrounding the area of implantation and enable cells attachment, proliferation differentiation and maintenance of cell function. Therefore, cells and biomaterials are the two main components of tissue engineering.

Tissue engineering scaffold is three dimensional structures that provide a site for cells to attach, proliferate, differentiate and secrete an extra-cellular matrix, eventually leading to tissue formation. The appropriate scaffold structure is also possible to guide cells into forming a tissue of predetermined, three dimensional shape and size. A scaffold cans be either permanent or temporary in nature, depending on the application and the function of the tissue. Temporary scaffold is made from biodegradable polymers, such as polyglycolic acid, polylactic acid and polycaprolactone which degrade within the body to leave a purely biological tissue. Permanent scaffold remains within the body, working with ingrown tissue to form a polymeric/biological composite (Edwards *et al.*, 2004).

Ideally, a scaffold should has the suitable characteristics for tissue regeneration (Table 2.1) such as (a) three dimensional structures with high porosity and an interconnected pore network for cell growth and the flow transport of nutrients and metabolic waste (b) biocompatibility with a controllable degradation and resorption rate to match the tissue growth (c) suitable surface chemistry for cell attachment, proliferation, and differentiation (d) mechanical properties to match those of the tissues at the site of implantation (Hutmacher *et al., 2000*).

Table 2.1 Ideal structural parameters of tissue engineering scaffold (Edwards *et al.*, 2004)

Scaffold function	Scaffold design parameter
Not to activate inflammatory response or toxicity *in vivo*.	Must be biocompatible, non-toxic and noncarcinogenic.
To assist in the growth of three dimensional tissue and organs.	Three dimensional scaffold of specific shape.
Give way to a uniform high cell seeding density.	High porosity and high interconnectivity between pores.
To provide the appropriate surface for cell attachment, proliferation and differentiation of function.	Optimum polymer surface chemistry and topography
To allow significant cell surface interactions such as cellular attachment.	High surface area to volume ratio.
To promote cell proliferation and migration leading to tissue growth throughout the scaffold.	Optimum pore size to allow for cell penetration, with high porosity and interconnectivity between pores.
To direct the orientation of cells, ECM and new tissue.	Correct fiber orientation within the scaffold.
To allow for the movement of nutrients and waste in and out of the scaffold.	High porosity and interconnectivity between pores.
The scaffold may degrade to leave only natural tissue.	Rate of degradation to match rate of tissue formation. Polymer degradation products must not be toxic or promote inflammation *in vivo*.
Possess sufficient structural integrity to retain shape *in vivo*, with enough mechanical strength to support developing tissue and withstand *in vivo* forces.	Scaffold should equal mechanical properties of developing tissue.

In the research of the bone tissue engineering can be classified into six phases. Design of bone tissue engineering should be three dimensional porous structures and fabricated from a highly biocompatible material which does not has

the potential to elicit an immunological. A polymer scaffold can be controlled the degradation rate at the same time as the specific tissue cells seeded into the scaffold. A scaffold is prepared from 3 types of the materials. The first is regulatory approved biodegradable and bioresorbable polymers (Table 2.2) such as collagen, polyglycolide (PGA), polylactides (PLLA, PDLA) and polycaprolactone (PCL). The second is a number of non approved polymers, such as polyorthoester (POE) and polyanhydrides which are also under investigation. The final is the synthesis of entrepreneurial polymeric biomaterials, such as poly (lactic acid co lysine) which can selectively shepherd specific cell phenotypes and guide the differentiation and proliferation into the targeted functional premature and mature tissue (Hutmacher *et al., 2000*).

In vivo, massive release of acidic degradation and resorption by products results in inflammatory reactions. If the capacity of the surrounding tissue to eliminate the by products is low, due to the poor vascularization or low metabolic activity, the chemical composition of the by products may lead to local temporary disturbances. Therefore, it is important that the dimensional scaffold-cell construct is exposed at all times to sufficient quantities of neutral culture media, especially during the period where the mass loss of the polymer matrix occurs.

Table 2.2 Properties of bioresorbable polymers (Hutmacher *et al., 2000*)

Polymer	Mechanical properties of polymer	Degradation process via hydrolysis	Loss of mechanical properties (month)	Mass loss (month)
Poly(L-lactide)	+++	Bulk erosion	9-15	36-48
Poly(L-lactide-co-D, L-lactide) 70/30	++	Bulk erosion	5-6	12-18
Poly(L-lactide-coglycolide) 10/90	++	Bulk erosion	1-2	3-4
Polyglycolide	+++	Bulk erosion	0.5-1	3-4

Poly(D,L-lactide)	+	Bulk erosion	1-2	5-6
Poly(D,L-lactideco-glycolide) 85/15	+	Bulk erosion	1-2	4-5
Poly(D,L-lactideco-glycolide) 75/25	+	Bulk erosion	1-2	4-5
Poly(D,L-lactideco-glycolide) 50/50	++	Bulk erosion	1-2	3-4
Polycaprolactone	+	Bulk and surface erosion	9-12	24-36
Polyorthoester	++	Surface erosion	4-6	12-18
Polyanhydrides	++	Surface erosion	4-6	12-18

+++ Good ++ Average + Poor

Control of the biochemical environment is essential for the successful in vitro engineering of three dimensional scaffold-tissue constructs for potential clinical use. Computer controlled bioreactors that continuously supply nutrients and gases, serve to regulate the required cell culture conditions for a long period of time. After the in vitro culturing of the three dimensional scaffold-tissue construct, the degree of remodeling and cell replacement of the bone transplant by the host tissue has to been taken into consideration. Cell and tissue remodeling is important for achieving stable mechanical conditions and vascularization at the host site. Hence, the three dimensional scaffold-tissue construct should maintain sufficient structural integrity during the in vitro and in vivo growth and remodeling process. The degree of remodeling depends on the host anatomy and physiology (Hutmacher *et al., 2000*).

2.1.2 Scaffold Manufacturing Methods

The method used to produce a scaffold determines the key properties of that scaffold, such as porosity, pore size and mechanical strength. When choosing the scaffold manufacturing method, it is important to take into consideration these desired scaffold properties, and to ensure that the method does not adversely affect these properties, e.g. mechanical characteristics or biocompatibility. Another consideration is the use of high temperatures and harsh chemicals during scaffold

manufacture, which can inhibit the incorporation of bioactive agents (e.g. growth factors) into the scaffold for drug delivery to the cells. Different manufacturing methods produce scaffolds of different configurations such as porous sponges and fibrous scaffolds (Figure 2.1) (Edwards *et al.*, 2004).

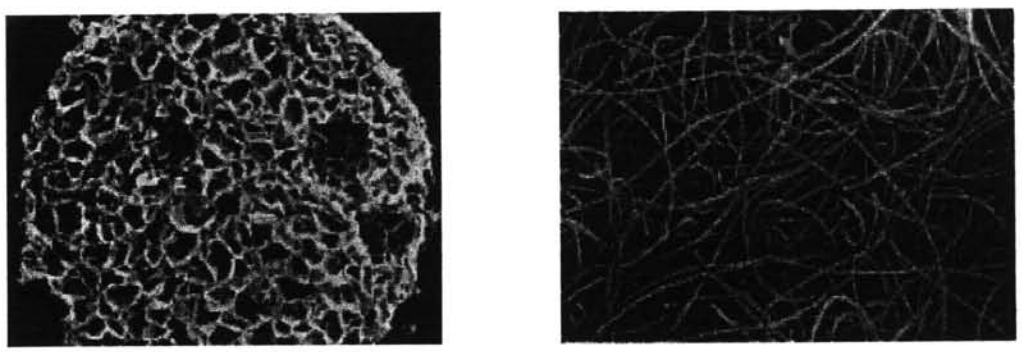


Figure 2.1 Sponge scaffold (left) and fibrous scaffold (right).

Skeletal tissue, such as bone and cartilage, is usually organized into three dimensional structures in the body. For the repair and regeneration of hard and ductile tissue, such as bone scaffolds need to have a high elastic modulus in order to be retained in the space they are designated for and provide the tissue with adequate space for growth. The scaffold material must have sufficiently high intermolecular bonding, but must have at the same time a physical and chemical structure which allows for hydrolytic attack and breakdown (Hutmacher *et al., 2000*).

Another point, which has to be focused on, is the diffusion of nutrients into the three dimensional scaffold. Although, an interconnected macropore structure of 300-500μm enhances the diffusion rates to and from the center of a scaffold, transportation of the nutrients and by products is not sufficient for large scaffold volumes. For bone tissue engineering, the creation of a vascularized bed ensures the survival and function of seeded cells, which have access to the vascular system for nutrition, gas exchange, and elimination of by products. The vascularization of a scaffold may be compromised by purely relying on capillary ingrowth into the interconnecting pore network from the host tissue. In situ, the distance between blood vessels and mesenchymal cells are not larger then 100 μm. Therefore, the time frame has to be taken into account for the capillary system to

distribute through larger scaffold volume. It may also be possible to control the degree and rate of vascularization by incorporating angiogenic and anti angiogenic factors in the degrading matrix of the scaffold (Hutmacher *et al., 2000*).

A number of fabrication technologies have been applied to process biodegradable and bioresorbable material into three dimensional polymeric scaffolds of high porosity and surface area. Table 2.3 summarizes the key characteristics and parameters of the techniques currently used (Hutmacher *et al., 2000*).

Table 2.3 Currently applied three dimensional scaffold fabrication technologies (Hutmacher *et al., 2000*)

Fabrication	Processing	Material properties required	Pore size (μm)	Porosity (%)	Architecture
Solvent casting and particulate leaching	Casting	Soluble	30-300	20-50	Spherical pores, salt particles remain in matrix
Membrane lamination	Solvent bonding	Soluble	30-300	<85	Irregular pore structure
Fabrication of non woven	Carding, Needling	Fibers	20-100	<95	Insufficient mechanical properties
Melt moulding	Moulding	Thermoplastic	50-500	<80	
Extrusion in combination with particular leaching	Extrusion through dies	Thermoplastic	<100	<84	Spherical pores, salt particles remain in matrix
Emulsion freeze drying	Casting	Soluble	<200	<97	High volume of inter-connected micropore structure
Thermally induced	Casting	Soluble	<200	<97	High volume of

phase separation					inter-connected micropore structure
Supercritical fluid technology	Casting	Amorphous	<100	10-30	High volume of inter-connected micropore structure
Supercritical fluid technology in combination with particle leaching	Casting	Amorphous	<50 <400	<97	Micropore structure combined with interconnected macropore structure
3-D printing in and without combination of particle leaching	Solid free form fabrication	Soluble	45-150	<60	100% interconnected macropore Structure
Fused deposition modeling	Solid free form fabrication	Thermoplastic	>150	<80	100% interconnected macropore structure

The solvent casting and particulate leaching method uses particulate porogens to form sponge like scaffolds. This method involves the dissolution of the polymer in an organic solvent, mixing with porogen, and casting the suspension into a predefined three dimensional mould. The solvent is subsequently allowed to evaporate. The main advantages of this processing technique are the ease of fabrication without the need of specialized equipment and manipulation of the scaffold pore size and porosity by altering the salt particle size and concentration respectively (Edwards *et al.*, 2004).

Gong *et al.* in 2006 prepared zein porous scaffold by the salt leaching method for bone tissue engineering. Zein is a major storage protein of corn. The molecular structure is a helical wheel confirmation with nine homologous repeating

units arranged in an anti-parallel form stabilized by hydrogen bonds. Zein has tough, glossy, hydrophobic, antioxidative and antimicrobial properties. The scaffolds possessed a total porosity of 75.3–79.0%, compressive modulus of 28.2–86.6 MPa and compressive strength of 2.5–11.8 MPa, the percentage degradation of 36% using collagenase and 89% using pepsin during 14 days incubation in vitro. The mechanical properties (i.e. compressive strength and modulus) of zein scaffolds were similar to those of cancellous bone, which implied a possible application on non load bearing areas. The morphology of pores located on the surface and within the porous scaffolds showed good pore interconnectivity. Rat mesebchymal stem cells (MSCs) could adhere, grow, proliferate and differentiate toward osteoblasts on porous zein scaffold.

2.1.3 Parameter Investigation

The better synthetic scaffold should promote tissue regeneration. Important factors include obtaining an optimal porosity and size of interconnecting but maintaining scaffold mechanical strength, enabling complete penetration of cells and nutrients throughout the scaffold, preventing the formation of necrotic tissue in the center of the scaffold. There are many researches to control the result the porosity, pore size and interconnecting size but maintaining mechanical strength. The scaffold structure including the size and morphology of the pores within the fabricated scaffold has the effect to allow osteoconduction and bone growth into the scaffolds whilst also allowing the transfer of nutrients through the scaffold. Generally, a pore size of greater than 100 μm but less than 400 μm is the optimum for bone cell growth into the scaffolds. Another important factor is the dimensions between the interconnecting pores as too small a pore can act to prevent osteoconduction through the scaffold structure. All of these factors affecting the porosity of the scaffolds must be considered and balanced with the mechanical strength of the scaffold. The requirement of tissue engineered scaffolds is that they must have sufficient mechanical strength for the application they are designed for. The microporosity within the scaffold has the effect to transfer of nutrients but also compromises the mechanical integrity of the struts within the scaffold (Cyster *et al.*, 2005).

Gong *et al.* in 2006 studied the effect of the size and the amount of porogen on the scaffold porosity, mechanical strength and degradation rate of the scaffold. They prepared zein porous scaffold by salt leaching method. Sodium chloride acting as porogen was used to generate an open pore structure. The mixture of zein and porogen was molded into cylindrical samples and then soaked in hot water (at 85 °C) for leaching. Pores are connected via the dissolving of salt porogen prior to formation of a three dimensional scaffold. Therefore, pores within the cross section of scaffolds using salt leaching process display a structure with favorable interconnectivity. Large macropores were developed in a range of 100–300 μm while much smaller micropores (tens of microns) were observed between the walls of macropores. The lack of organized pore structures is likely due to the good interconnectivity of salt leached samples, which reduced the presence of well organized, largely closed off pores. Highly interconnective pores are useful in bone tissue engineering for achieving vascular in growth to maximum depths within a scaffold system. They found that scaffolds fabricated with larger porogen size after leaching formed larger pores on the surface scaffold (Figure 2.2). However, it seemed that the porogen size had no significant effect on the characteristics of inner pores within zein scaffolds. The porogen amount (at the same particle size) had significant influence on the porosities of scaffolds, but the porogen size (at the same amount) had no significant effects on the porosities of scaffolds. The effects of porogen size and amount sodium chloride on Young's modulus (MPa) and compressive strength (MPa) of scaffolds were displayed. When the amount of porogen increased, both compressive strength and modulus will be decreased. In addition, the higher porosity corresponded to the lower Young's modulus and the lower compressive strength, which was consistent with the inverse tendency between porosity and mechanical properties. Degradation rate of the scaffold increased with porosity of the scaffold. These results indicated that a greater amount of porogen leads to a higher porosity that contributes to the faster degradation of scaffolds.

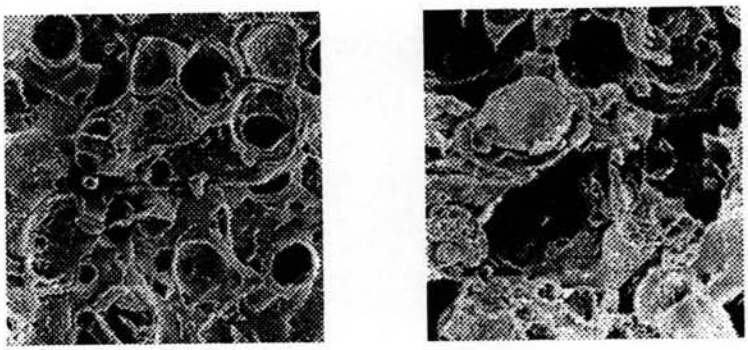


Figure 2.2 Scaffold fabricated porogen size of 38.5-75 μm (left) and 150-220 μm (right).

2.2 Bone Tissue Engineering

Bone is an amazing and a true nanocomposite. It is a complex and a highly specialized form of connective tissue involve to the formation of the skeleton of the body. Bone, not only provides mechanical support but also elegantly serves as a reservoir for minerals, particularly calcium and phosphate. It is a good example of a dynamic tissue, since it has a unique capability of self regenerating or self remodeling to a certain extent throughout the life without leaving a scar. The main compositions of the bone are organic (protein: collagen) and inorganic (mineral: hydroxyapatite) phase. An overall composition of the bone is given in Table 2.4. The bone mineral is mainly composed of HAp and the bone protein is mainly composed of collagen. Here, collagen acts as a structural framework in which plate like tiny crystals of HAp are embedded to strengthen the bone. The bone collagen has a typical fibrous structure, whose diameter varies from 100 to 2000 nm. Similarly, HAp in the bone mineral is in the form of nanocrystals, with dimensions of about 4nm to 50nm. The bone minerals are also enriched with a few trace elements for various metabolic functions, which include carbonate, citrate, sodium, magnesium, fluoride, chloride, and potassium. The prime role of minerals is to provide toughness and rigidity to the bone, whereas collagen provides tensile strength and flexibility. Nature has built extremely hard and tough bone using such soft (collagen) and brittle (HAp) ingredients (Murugan *et al.*, 2005).

Table 2.4 The composition of bone (Murugan *et al.*, 2005)

Inorganic phase	wt%	Organic phase	wt%
1. Hydroxyapatite	60	1. Collagen	20
2. Carbonate	4	2. Water	9
3. Citrate	0.9	3. Non-collagenous proteins (osteocalcin, osteonectin, osteopontin, thrombospondin, morphogenetic proteins, sialoprotein, serum proteins)	3
4. Sodium	0.7	4. Other traces: polysaccharides,	
5. Magnesium	0.5	lipids, cytokines	
6. Other traces: Cl^-, F^-, K^+ Sr^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}		Primary bone cells: osteoblasts, osteocytes, osteoclasts.	

The key to the strength of the bone is the complex structural hierarchy into which it is organized in a self assembling mode. It is important that the minerals are not directly bound to collagen, but bound through non collagenous proteins. The non collagenous proteins have approximately 3–5% of the bone, which provide active sites for biomineralization and for cellular attachment. Water is also found in sufficient quantity in all the bones. It is one of the most essential substances of the body because no cells survive without water. The amount of water present in the bone is an important determinant of its mechanical behavior. Biomechanical properties of the bone are given in Table 2.5. Lipids are also necessary for the cellular functions, which account to about 2% of the bone. They play an important role in the process of initial biomineralization. The degree of biomineralization is the most important factor to determine the biomechanical competence of the bone (Murugan *et al.*, 2005).

Table 2.5 Biomechanical properties of bone (Murugan *et al.*, 2005)

Properties	Cortical bone	Cancellous bone
Young modulus (GPa)	14-20	0.05-0.5
Tensile strength (MPa)	50-150	10-20

Compressive strength (MPa)	170-193	7-10
Fracture toughness (MPa $m^{1/2}$)	2-12	0.1
Strain to failure	1-3	5-7
Density (g/cm^3)	18-22	0.1-1.0
Apparent density (g/cm^3)	1.8-2.0	0.1-1.0
Surface / bone volume (mm^2/mm^3)	2.5	20
Total bone volume (mm^3)	$1.4x10^6$	$0.35x10^6$
Total internal surface	$3.5x10^6$	$7.10x10^6$

The cancellous bone has about 20% of the total bone. It is the spongy bone. It is lighter and less dense than compact bone (Figure 2.3). It has high porosity and higher concentration of blood vessels compared to compact bone. The porous architecture is easily visible under lower power microscopes and even to the naked eye if the pores are very large. The diameter of the pores may be from few micrometers to millimeters. The cortical bone is much denser than spongy bone. It is the compact bone. It has about 80% of the total bone. It has less porosity and thus less concentration of blood vessels. Its porous architecture is not visible to naked eye. The pores may be 10–20 μm in diameter and mostly separated by intervals of 200–300 μm. The cortical bone functions mechanically in tension, compression, and torsion, whereas cancellous bone functions mainly in compression. At the microstructural level, the repeated structural unit of cortical bone is mostly of osteon, which act as weight bearing pillars. The cancellous bone is made of an interconnecting framework of trabeculae. At the nanostructural level, the bone is comprised mainly of collagen fibers and nanocrystals of bone minerals, particularly HAp (Murugan *et al.*, 2005).

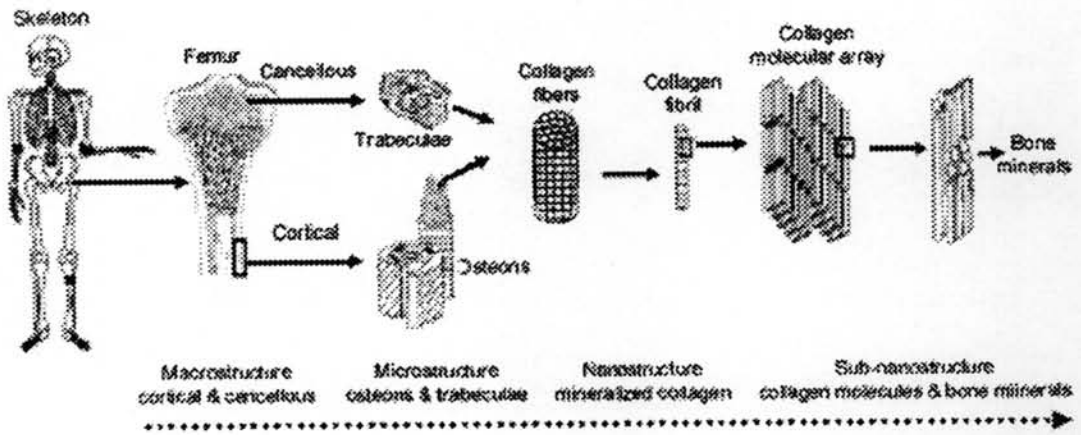


Figure 2.3 The hierarchical structure of bone, from macro to nano assembly (Murugan *et al.*, 2005).

Polymers which are widely used in bone tissue engineering should be biocompatibility, design flexibility, functional groups availability, surface modifiability, light weight, and ductile nature. Polymers can be degraded by cellular or enzymatic pathways that undergo hydrolysis upon exposure to the bodies aqueous environment. The rate of biodegradation can also be controlled by manipulating the polymer properties such as hydrophobicity and crystallinity. Using the composite approach, it is possible to manipulate the mechanical properties such as strength and modulus of the composites closer to natural bone with help of secondary substitution phases. For example, HAp-polymer composites have an elastic modulus near to that of bone and are more mechanically reliable than their monolithic constituents. A graphical representation of mechanical consistency of various HAp-based composites is given in Figure 2.4 in comparison with a natural bone (Murugan *et al.*, 2005).

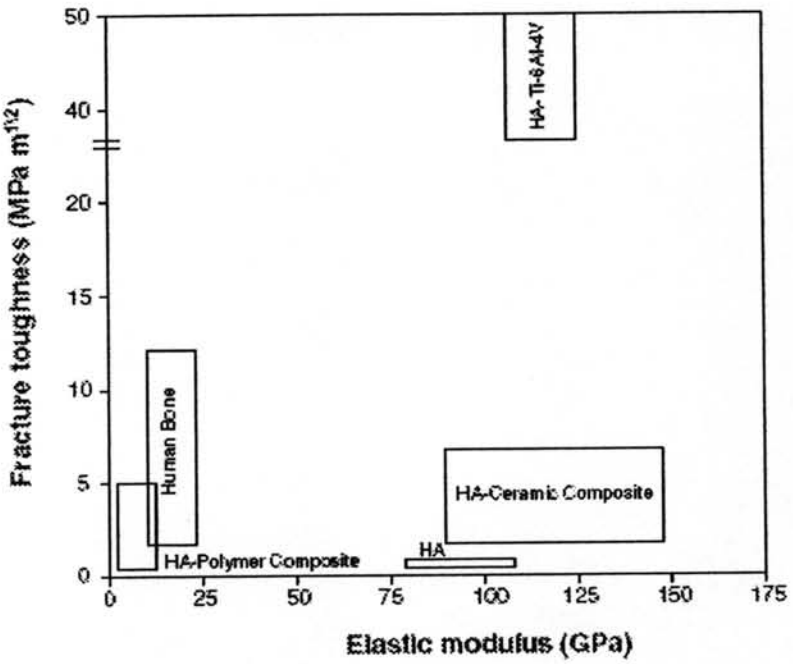


Figure 2.4 A graphical representation of relationship between toughness and modulus of various HAp-based composite materials (Murugan *et al.*, 2005).

The prime concept of tissue engineering is to isolate a small biopsy of specific cells from a patient, to allow them to culture on scaffold, to transplant the cell engineered scaffold into the defective site of the patient's body that needs bone regeneration, and to guide or direct new tissue formation into the scaffold that can be biodegraded over time. Three key factors have to be considered for the success of bone tissue engineering. They are cells, scaffold, and cell matrix (scaffold) interaction. The scaffold plays a pivotal role in accommodating the cells. These cells then undergo proliferation, migration, and differentiation, leading to the formation of a specific tissue. Scaffolds loaded with growth factor have regulated cellular growth and related functions in a better way. Bone tissue engineering approach to treat bone defects must involve the use of osteoconductive scaffold with osteogenic cells and osteoinductive growth factors. Figure 2.5 shows a design strategy for a tissue engineered nanocomposite. As HAp is an osteoconductive agent, it can be used as a scaffold matrix for bone tissue engineering. However, it does not possess osteoinduction ability and its biodegradability is also relatively slow. To circumvent

these drawbacks, biodegradable polymers can be employed to make a composite with HAp (Murugan *et al.*, 2005).

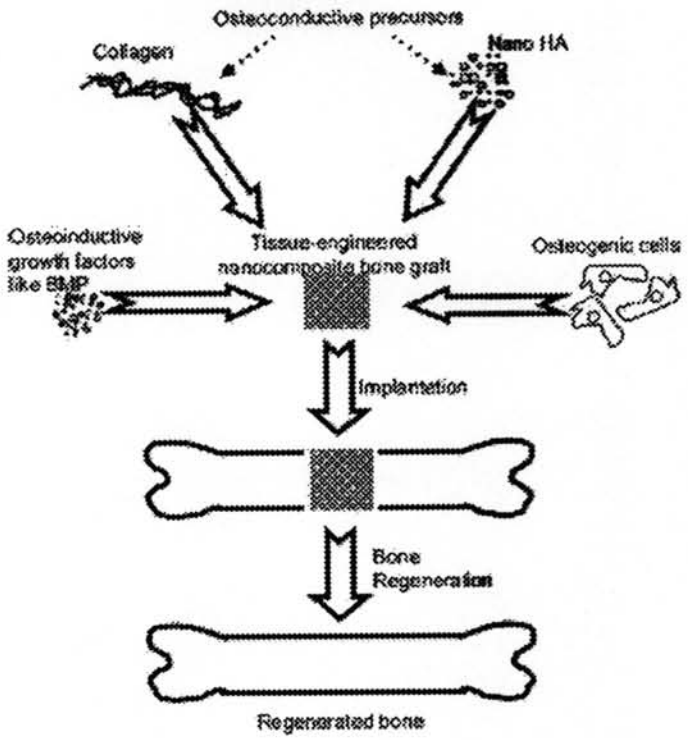


Figure 2.5 Design strategy of tissue engineered nanocomposite bone substitute (Murugan *et al.*, 2005).

2.3 Hydroxyapatite (HAp)

Hydroxyapatite is a class of calcium phosphate based bioceramic. It has a chemical and structural similarity with natural bone mineral. HAp has a chemical composition of $Ca_{10}(PO_4)_6(OH)_2$ with Ca/P ratio of 1.67. The HAp derived either from natural sources or from synthetic sources is regarded as bioactive substance, since it forms a strong chemical bond with host bone tissue. HAp is not only bioactive but also osteoconductive, non-toxic, non-immunogenic, and its structure is crystallographically similar to that of bone mineral with adequate amount of carbonate substitution. The properties of HAp are given in Table 2.6, which makes HAp an appropriate bone graft material (Murugan *et al.*, 2005).

Table 2.6 Physicochemical, mechanical, and biological properties of HAp (Murugan *et al.*, 2005)

Properties	Experimental data
Chemical composition	$Ca_{10}(PO_4)_6(OH)_2$
Ca/P molar	1.67
Crystal system	Hexagonal
Space group	$P6_3/m$
Cell dimensions (A˚)	a = b = 9.42, c = 6.88
Young's modulus (GPa)	80–110
Elastic modulus (GPa)	114
Compressive strength (MPa)	400–900
Bending strength (MPa)	115–200
Density (g/cm^3)	3.16
Relative density (%)	95–99.5
Fracture toughness ($MPa\ m^{1/2}$)	0.7–1.2
Hardness (HV)	600
Decomposition temperature (°C)	>1000
Melting point (°C)	1614
Dielectric constant	7.40–10.47
Thermal conductivity (W/cm K)	0.013
Biocompatibility	High
Bioactivity	High
Biodegradation	Low
Cellular compatibility	High
Osteoinduction	Low
Osteoconduction	High

The interactions of osteogenic cells with bioceramics are important for bone regeneration. Bioactive ceramics are known to enhance osteoblast differentiation as well as osteoblast growth. However, their applications have been limited because of their brittleness, difficulty of shaping, and an extremely slow degradation rate in the case of HAp. The use of biodegradable polymer-bioceramic

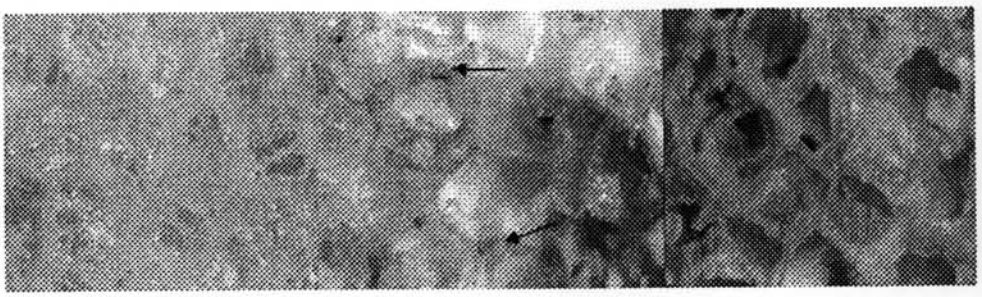
composites could be a solution to this problem. The addition of biodegradable to calcium phosphate ceramics would allow for better manipulation and control the structure of the composites to fit bone defects and reduce the brittleness of the ceramics (Kim *et al.*, 2006).

Kothapalli *et al* in 2005 prepared scaffolds comprising poly(lactic acid) and nanohydroxyapatite (HAp) using the solvent casting and salt leaching technique. NaCl was used as the leaching agent. Nanosized HAp was synthesized by a hydrothermal method at 170°C and autogenous pressure. The particles had an average size of approximately 25 nm in width and 150 nm in length. HAp/PLA three dimensional scaffolds with porosity 90% and interconnectivity were fabricated. As the HAp content increased in the scaffold from 0 to 50 wt%, the compression modulus of the scaffolds increased from 4.72 ±1.2 to 9.87 ± 1.8 MPa, while the yield strength from 0.29 ±0.03 to 0.44 ±0.01 MPa. It was observed in this study that HAp was homogenously distributed in the polymer scaffold when the loading was low (up to 40 wt %), but uneven distribution occurred when the loading was above 40 wt%. The uneven distribution of HAp would probably adversely affect the mechanical properties of the scaffold. Thus the HAp loading in the current study was constrained to below 50 wt% in the scaffold. Such polymeric scaffolds should be suitable materials for non load sharing tissue engineering applications.

Wang *et al* in 2005 blended hydroxyapatite (HAp) into poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) to make films and scaffolds. After HAp blending, mechanical properties of PHB including compressive elastic modulus and maximum stress showed improvement and osteoblast responses including cell growth and alkaline phosphatase activity were also strengthened. HAp effect to the surface smoothness on both of PHB and PHBHHx scaffolds. As osteoblast preferred surfaces with appropriate roughness, this may explain for the fact that PHB/HAp and PHBHHx scaffolds with appropriate roughness showed the strongest biocompatibility compared with PHB and PHBHHx/HAp scaffolds, which were more porous on the surfaces. Crystallinity of PHB and PHBHHx may also affect exposure of HAp particles on surface. After blending with HAp, PHB with higher crystallinity may

exclude more HAp on surface compared with the amorphous PHBHHx. As exposed HAp particles should be favourable for focal contact formation of osteoblasts, this may be the reason why blending with HAp into PHB has better effect on promoting osteoblast growth than that into PHBHHx. More aggregations of HAp particles (phase separation) were observed on PHBHHx/HAp scaffold than that on PHB/HAp scaffold which may contribute to the result that introduction of HAp particles into PHB increased mechanical properties while that into PHBHHx had adverse effect. This indicates that PHB may be more compatible with HAp compared with PHBHHx. From these results, it was suggested that osteoblast responses to HAp blending with PHB may be related with surface morphology, surface chemistry changes resulted from the presence of HAp particles. For PHBHHx, though HAp exposed on surface promoted osteoblast anchorage, a too smooth surface of PHBHHx/HAp was not favorable for osteoblast growth. Although HAp is bioactive and osteoconductive, HAp blending with PHBHHx reduced the porosity of PHBHHx surface, thus resulted in reduced osteoblast proliferation.

Kim *et al* in 2006 fabricated Poly(D,L-lactic-co-glycolic acid)/nano-hydroxyapatite (PLGA/HAp) composite scaffolds by the gas forming and particulate leaching (GF/PL) method without the use of organic solvents and solvent casting / particulate leaching (SC/PL). Since the SC/PL and GF/PL scaffolds have similar physical properties such as porosity, pore size, and interconnectivity, the difference in osteogenic ability between the two scaffold types might be due to their different surface chemistries (Figure 2.6). Nanosized HAp particles have a high surface area, to fabricate the composite scaffolds, improve bioactivity and osteointegration when implanted in the bone defect sites and enhance the protein adsorption and cell adhesion.



(A) (B) (C)

Figure 2.6 (A) Microscopic images of PLGA no HAp GF/PL
(C) Microscopic images of PLGA/HAp SC/PL(The arrows indicate HAp particles.)
(D) Microscopic images of PLGA/HAp GF/PL.

Chen *et al* in 2005 prepared hydroxyapatite reinforced poly(ε-caprolactone) composites with different HAp sizes and molecular weight distributions of PCL using a melt processing method. They found that the HAp size and the molecular weight distribution of PCL affected the interaction between PCL and HAp and the properties of the composites (Figure 2.7). The HAp with smaller particle sizes had greater improvement in yield strength, tensile modulus, storage modulus and loss modulus, due to its larger surface area and stronger interaction. The PCL with broader molecular weight distribution had weaker interaction and behaved some what different from that with narrower distribution in that its low molecular weight fraction contributed more to elastic property.

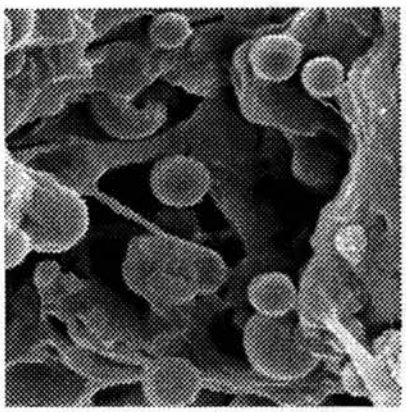


Figure 2.7 SEM micrographs for 20% PCL/HAp composites by melt processing.

2.4 Polycaprolactone (PCL)

PCL is also an important member of the aliphatic polyester family. It has been used to effectively entrap antibiotic drugs and considered as a drug delivery system, being used to enhance bone ingrowth and regeneration in the treatment of bone defects. The degradation of PCL proceeding in two stages includes random hydrolytic ester cleavage and weight loss through the diffusion of oligometric species from the bulk. It has been found that the degradation of PCL system with a high molecular weight (M_n of 50,000) is remarkably slow, requiring 3 years for complete removal from the host body (Rezwan *et al.*, 2006).

The propensity of ε-caprolactone to undergo ring opining polymerization was first estabilished by Carothers during the early 1930s. Poly(ε-caprolactone) (PCL) as a biodegradable polymer is degraded by microorganisms in the environment. The homopolymer itself is degraded very slowly when compared with polyglycolic acid and polyglycolic acid co lactic acid, and is most suitable for long term implant systems. This fact, coupled with a high permeability to many therapeutic drugs and a lack of toxicity, has make PCL and its derivatives well suit for using in medical applications. Polymerization of ε-caprolactone can be effected by at least four different mechanisms including anionic, cationic, coordination and radical polymerization. Each method has unique attribute, providing different degrees of control of molecular weight and molecular weight distribution, end group composition and the chemical structure and sequence (block versus random) distribution of copolymers. Each of these characteristics is important in defining the permeability and degradability of the polymer.

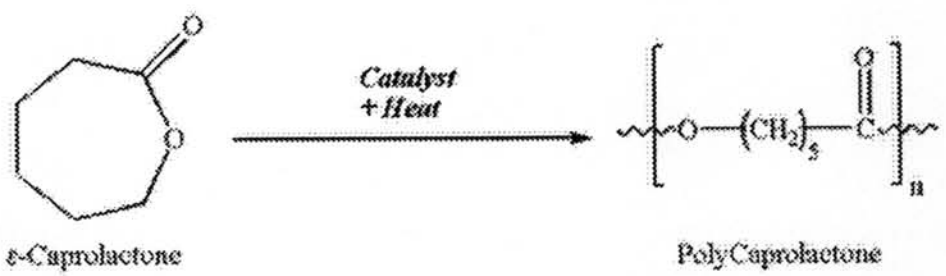


Figure 2.8 Ring opening polymerization of caprolactone (http://en.wikipedia.org/).

2.4.1 Polymer Properties

Poly (ε-caprolactone) (PCL) is a semicrystalline polymer, melting in the range of 59-64°C, depending on the crystallite size. Because of its low glass transition temperature (T_g) of -60°C, the melt cannot be quenched to a glass. The heat of fusion (ΔH_f) of 100% crystalline PCL is reported to be 139.3 J/g, a value that has been used to estimate the crystallinity of PCL and its copolymers from differential scanning calorimetry (DSC) traces. The crystallinity of PCL varies with its molecular weight. For molecular weights in excess of 100,000 the crystallinity is about 40%, rising to 80% as the molecular weight decreases to 5,000. Crystallinity is known to play an important role in determining both permeability and biodegradability because of the generally accepted fact that the bulk crystalline phase is inaccessible to water and other permeates. That is, an increase in crystallinity reduces the permeability by both reducing the solute solubility and increasing the tortuosity of the diffusion pathway. The biodegradation rate is reduced by the decrease in accessible ester bonds.

The solubility parameters of PCL are 20.8 and 20.4 $J^{1/2}cm^{-3/2}$ when calculated using the parameters of Fedors and Hoy, respectively. PCL is soluble in a number of solvents at room temperature, including THF, chloroform, methylene chloride, carbon tetrachloride, benzene, toluene, cyclohexanone, dihydropyran, and 2-nitropropane. It is poorly soluble in acetone, 2-butanone, ethyl acetate, acetonitrile, and DMF, and insoluble in alcohols, petroleum ether, and diethyl ether.

2.4.2 Biodegradation of Poly (ε-caprolactone) (PCL)

PCL can be degraded by random hydrolytic chain scission of the ester linkages, manifested by a reduction in the viscosity and molecular weight of the polymer. There is no weight loss during this first stage of the degradation process.

The second phase of polymer degradation is characterized by a decrease in the rate of chain scission and the onset of weight loss. Weight loss has been attributed to the increased probability that chain scission of a low molecular weight polymer will produced a fragment small enough to diffuse out of the polymer bulk and the breakup of the polymer mass to produce smaller particles with an increased probability of phagocytosis. The rate of chain scission of polyesters can be

retarded by end capping to reduce the initial carboxylic acid end group concentration. Alternatively, the rate may be increased by acidic additives that supplement the effect of the carboxyl end groups.

2.4.3 Medical Application of Polycaprolactone

Williamson *et al.*, 2005 fabricated poly (ε-caprolactone) (PCL) fibers by wet spinning from solutions in acetone under low shear (gravity flow) conditions. Cold drawing to an extension of 500% resulted in increases in fiber strength (43 MPa) and stiffness (0.3 GPa) and development of an oriented, fibrillar surface texture. Gelatin was modified on the PCL fibers. Proliferation of fibroblast and myoblast cell types was consistently higher on gelatin coated fibers relative to as spun fibers at time points below 7 days. Fibroblast growth rates on cold drawn PCL fibers exceeded those on as spun fibers but myoblast proliferation was similar on both substrates. The high fiber compliance combined with a potential for modifying the fiber surface chemistry with cell adhesion molecules and the surface architecture by cold drawing to enhance proliferation of fibroblasts and myoblasts.

Hattori *et al.*, 2001 prepared a blended thread by mixing poly-L-lactic acid (PLLA) and polycaprolactone (PCL) fiber for bone fixation by melting. The fatigue strength of this thread was higher than that of a stainless-steel wire with the same cross-sectional area. It can be used as the new material for bone fracture in the future.

2.5 Polycaprolactone and Hydroxyapatite Composites

Composite comprising poly (ε-caprolactone) (PCL) and hydroxyapatite (HAp) has found applications in the substitution, regeneration, and repair of bone tissues and other orthopedic usage. Hydroxyapatite takes the form of coarse particles, which resemble the apatite in the natural bone. Poly (ε-caprolactone) is a hydrolytic polyester having appropriate resorption period and releases nontoxic byproducts upon degradation (Baji *et al.*, 2006).

Azevedo *et al.*, 2003 fabricated PCL-HAp composites with enhanced interfacial interaction between the polymer and the reinforcement material for

increasing the composite mechanical properties. They blended HAp particles (particles size 38-53μm) with PCL (M_w =80,000) in an extruder at 130°C for 30 min and found that the grafting of PCL on the surface of the HAp particles lead to a better PCL-HAp interface and consequently to move hydrolytically stable composites. The modulus increased with an increase in filler concentration. The observed decrease in tensile strength was attributed to non-optimization of the HAp-PCL interfaces.

Novel bone scaffolding materials fabricated by electrospinning from polycaprolactone (PCL) solutions containing nanoparticles of calcium carbonate ($CaCO_3$) or hydroxyapatite (HAp), had been described by Wutticharoenmongkol *et al.*, 2005. The diameters of the as spun fibers were found to increase with an increase in the amounts of nanoparticles and caused to increase in the tensile strength of the obtained fiber mats. An increase in the concentration of the base PCL solution caused the average diameter of the as-spun PCL-HAp composite fibers to increase. Increasing applied electrical potential also resulted in an increase in the diameters of the obtained PCL-HAp composite fibers. The electrospun mats of PCL, PCL-$CaCO_3$, and PCL-HAp fibers were evaluated the cytotoxicity based on human osteoblasts (SaOS2) and mouse fibroblasts (L929).They revealed that these as-spun mats posed no threat to the cells. In 2006, they evaluated in vitro with human osteoblasts (SaOS2) in terms of attachment, proliferation, and alkaline phosphatase (ALP) activity of the cells by cultured directly on the scaffolds. The results were compared with those on corresponding solution-cast film scaffolds and tissue-culture polystyrene plate (TCPS). It was found that all of the fibrous scaffolds promoted much better adhesion and proliferation of cells than the corresponding film scaffolds and TCPS. Interestingly, the cells that were seeded on all of the fibrous scaffolds appeared to be well expanded and attach on the fiber surface very well even only about 1 h in culture, while those seeded on all of the film scaffolds and the glass substrate were still in round shape. Among the various fibrous scaffolds investigated, the one that was filled with 1.0% HAp showed the highest ALP activity. Finally, all of the fibrous scaffolds exhibited much greater tensile strength at yield than all of the corresponding film scaffolds.

Chen *et al.*, 2005 prepared the PCL-HAp biocomposite by blending in melt form at 120°C. It was observed that the composite containing 20wt% HAp had the

highest strength. The tensile modulus of the scaffold increased with an increase in the concentration of HAp. The mechanical properties of the scaffold depended on the conjoint and mutually interactive influences of the molecular weight of the polymer and the particle size of the hydroxyapatite. A higher yield strength and modulus was observed for smaller particles of HAp. This finding is interesting and ascribed to the larger interfacial surface area of the smaller HAp particles. Both the crystallization temperature (T_c) and melting temperature (T_m) were found to increase with an increase in the concentration of HAp, suggesting good interaction between PCL and HAp. The PCL matrix with a narrow molecular weight was observed to have stronger interfacial interaction and greater T_c and T_m.

Marra *et al.*, 1999 prepared biocomposite by dissolving polycaprolactone in chloroform at room temperature (7–10% w/v). Sieved NaCl (150–250mm particle size), and HAp (10mm particle size) were suspended in the solution and sonicated for 60 s. The solvent was evaporated and the scaffold was subsequently immersed in distilled water at room temperature for 24 h. The scaffolds were later dried and cut into discs of 12mm in diameter. The incorporation of HAp was studied using 0–50% of HAp (w/w). Porosity was as high as 80% and controlled by the amount of NaCl. The mechanical properties obtained using this technique was just one-third that of trabecular bone. Both the porosity and pore size resulting from this method can be matched with that of the trabecular bone, which allows for the regeneration of structurally equivalent trabecular bone within the biomaterial.

Controlled biodegradation is a critical factor in developing tissue scaffolds that can be gradually reabsorbed by and excreted from the body. Biodegradability generally depends on the following factors including chemical stability of the polymer backbone, hydrophobicity of the monomer, morphology of the polymer, initial molecular weight, fabrication processes, geometry of the implant, and properties of the scaffold such as porosity and pore diameter. Biodegradation refers to a gradual chemical breakdown of the polymer as a result of the action of hydrolytic reaction and living organisms. The result is reflected in a noticeable change in mechanical, physical, and other material properties. Weight loss and degree of water uptake provides an indication of degradation of the composite. This can be calculated using the relationship

$$\%S = (M_a - M_i)/ M_i \times 100$$

$$\%W = (M_i - M_d)/ M_i \times 100$$

where %S is the degree of water uptake, %W the weight loss, M_a the weight of the sample after immersion in the solution, M_i the initial weight of the sample, and M_d the weight of the sample at the end of the experiment, after drying to achieve constant weight. Mechanical properties of the composite can be characterized by loading the sample on a universal testing machine and obtaining the stress-strain relationships. It was observed that modulus increased with an increase in HAp concentration. Improving the interaction between HAp and PCL coupled with good mixing of HAp and PCL will enhance the mechanical properties of the composite. The mechanical properties of the composite and the bone counterparts are given in Table 2.7. The mechanical properties of the scaffold depends on the interactive parameters such as pore size, porosity, processing methodology used, particle size of the HAp, and distribution of the molecular weight of the PCL matrix. The mechanical property of the tissue scaffold can be controlled by (a) varying the compositions of components in the scaffold, (b) inducing porosity by microspheres, and (c) control of pore size using emulsion techniques. Carefully adjusting the molecular weight of the polymers can also alter the degradation rates. These are attributes of the PCL/HAp composites and they are potentially useful for tissue engineering (Baji *et al.*, 2006).

Table 2.7 Comparison of mechanical properties of bones, HAp, PCL, and scaffold materials

	Ultimate tensile strength (MPa)	**Young's modulus(MPa)**	**Elongation (%)**
Bone - Parallel - Normal	 124-174 49	 17.0-18.9 x 10^3 11.5 x 10^3	
Trabecular bone	8	50-100	
HAp >99.2% dense		80-100 x 10^3	
PCL	10.12±0.97	158.59±11.48	438.36±26.04
PCL/HAp composite			

(wt %)			
- 10	3.72±0.33	91.98±7.11	17.25±1.08
- 20	2.81±0.23	106.35±5.81	7.49±0.54
- 30	2.53±0.21	111.92±3.97	6.76±0.43
- 40	2.39±0.14	118.41±2.59	5.77±0.42

2.6 Ipriflavone

An ideal scaffold requires three dimensional porous interconnected structure to facilitate cell growth and transport of nutrients and metabolic waste, biocompatible and bioresorbable with controlled degradation and resorption rates, mechanical properties quite similar to those of the neighboring tissues, an intrinsic capability that would permit cell attachment and proliferation. Tissue growth and cellular behavior can be promoted by a judicious choice of manufacturing processes and the use of growth factors in the scaffolds (Baji *et al.*, 2006).

Ipriflavone is a synthetic derivative of the plant isoflavone, which is primarily found in soy products. Isoflavones belong to a larger category known as flavonoids, which are natural plant components that have antioxidant, anti-inflammatory, anti-allergy, and anticancer properties. Ipriflavone contains three carbon rings. Its chemical names are 7-isopropoxyisoflavone, 7-isopropoxy-3-phenyl-4H-1-benzopyran-4-one, 7-(1-methylethoxy)-3-phenyl-4H-1-benzopyran-4-one and 7-isopropoxy-3-phenylchromone. Ipriflavone is abbreviated as IP. It is a solid substance that has poor solubility in water. It has been approved for the treatment of involutional osteoporosis in some European countries and in Japan. It has been widely studied in humans and found effective for inhibiting bone resorption and enhancing bone formation, the net result being an increase in bone density and a decrease in fracture rates in osteoporotic.

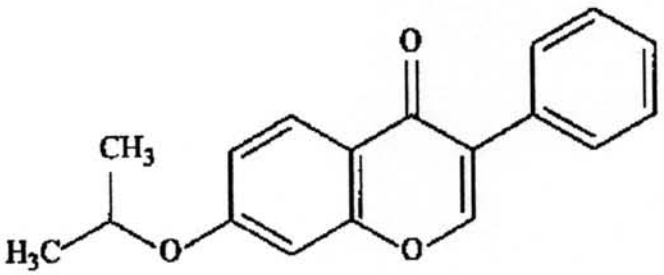


Figure 2.9 The structural formula of ipriflavone.

Perugini *et al.*, in 2002 designed a film dosage form for sustained delivery of ipriflavone into the periodontal pocket. They prepared monolayer composite systems made of ipriflavone loaded poly(D,L-lactide-co-glycolide) (PLGA) micromatrices in a chitosan film form by emulsification/casting/evaporation technique. They found that the PLGA microspheres containing ipriflavone have been formed and entrapped in the chitosan matrix. The emulsification process allowed to produce films composed of a homogeneous dispersion of a lipophilic drug into a hydrophilic polymer such as chitosan. The chitosan-PLGA films showed "in vitro" drug release profiles suitable to a prolonged therapy. Ipriflavone release depends not only on diffusion through PLGA micromatrices, but also on diffusion through chitosan matrix. The micromatricial structure of films studied in their work offer the possibility of obtaining polymeric systems with good morphological characteristics, such as thickness and flexibility useful for periodontal pocket delivery.

Martini *et al.*, 1998 studied the effect of ipriflavone (IP) on *in vivo* bone formation in rat perialveolar bone by surgically producing a hole in the mandibular bone. The holes were filled with powdered IP. Morphological measurements of the areas occupied by new bone showed that the synthesis of perialveolar bone was significantly stimulated by IP. In conclusion, IP can stimulate osteogenesis and suggest that this compound could represent a potential therapeutic tool to promote repair of injured perialveolar bone.