CHAPTER 2

LITERATURE REVIEW

2.1 Overview on bone

Bone is a dynamic organ that put together part of the endoskeleton of vertebrates. It is produced by the deposition of minerals in our body. Bone has a different organization of structures at a different length scales which work to perform various function such as structural support, protection and storage of healing cells, and mineral ion homeostasis (Liu and Webster, 2007).

Bone can be divided into three levels of structure depending on the size:

1. The nanostructure such as non-collagenous organic proteins, fibrillar collagen and mineral crystals.
2. The microstructure such as lamellae, osteons and Haversian systems.
3. The macrostructure such as cancellous and cortical bone.

2.1.1 Nanostructure of bone

The component of this hierarchical architecture level is the building blocks of bone which composed of both organic compounds and inorganic compounds. The main structures observed at this level would be the collagen fibers which are surrounded and infiltrated by minerals. The exact composition of bone varies

depending on factors such as species, age, dietary profile, health status and the location of the bone.

Almost 90 % of the organic phase is made up of Type I collagen while the remaining is made up of noncollagenous proteins and ground substances. Type I collagen is synthesized by osteoblasts. The linear molecules of Type I collagen are self-assembled in the form of triple helix bundles with a specific periodicity and gaps which are called hole-zones situated between the ends of the molecules and also between the sides of the parallel molecules. These fibers accommodate the framework and architecture of bone. Hydroxyapatite (HA) crystals can be found located between the fibers.

On the other hand, non-collagenous proteins, bone inductive proteins and extracellular matrix compounds made up the 10% of the organic phase. Non-collagenous proteins are produced by osteoblasts during new bone formation. At the same time, calcium and phosphate ions are deposited in between the hole-zones and pores of the collagen matrix to promote the growth of HA crystals. Despite the small percentage of contribution to the overall weight of the bone, they have major contributions to bone's function. Nevertheless, the ground substances can be found filling up the spaces between the collagen fibers and HA crystals.

The HA crystals is the inorganic component of bone. As mentioned earlier on, it is located between the collagen fibers. The location of these crystals limits the possible growth of mineral crystals, forcing it to be discrete and discontinuous. The size of these crystal plates are approximately 50 × 25 nm and the thickness is about 2-3 nm (Rho *et al.,* 1998). Small amount of impurities may

be present in the mineralized HA matrix. These impurities might affect the cellular functions of bone such as altering certain physical properties of bone and important biological aspects which are crucial for normal function of the bone (Liu and Webster, 2007).

2.1.2 Microstructure of bone

Bone consists of two main structures in this level. They are woven and lamellae. Woven bone is located in the metaphyseal region of growing bone, in fracture callus and in diseased bone. Woven bone is in a disorganized structure and it is made up of disoriented coarse collagen fibers, but forms quickly.

Lamellae bone is the resultant of remodelling of woven bone or existing bone. It is stronger and highly organized. It is filled with collagen fibers which lie in parallel in each lamella and this explained the properties of having a great strength which is parallel to the longitudinal axis of the collagen fibers. Lamellae bone forms concentric rings called osteons with a central blood supply known as the Haversian system (Liu and Webster, 2007).

2.1.3 Macrostructure of bone

At this level, bone is divided into two types: cortical bone or compact bone and cancellous bone or spongy bone. Cortical bone is densely packed (less than 30% porosity) and is composed of small pores up to 1 mm in diameter. It covers about 80 % of all skeletal tissue and it has a structural form suitable to withstand stress. The cortical bone surrounds the cancellous bone, which is made up of intermeshing thin, bony plates that fill the bony cavities and are in contact with

the bone marrow. Cancellous bone exposes a large area to the body fluid. It is characterized by a sponge-like branching network structure with 50 to 90 % porosity and large pores with the size of several millimetres in diameter. Cancellous bone is more active and thus it shares a greater extent than cortical bone by changes in skeletal metabolism (Liu and Webster, 2007).

2.2 Bone cells and bone remodelling

Bone being a dynamic organ has the ability to regenerate when damaged and to remodel when the loading condition change. Therefore the definition of bone remodelling can be best described as the process of resorption followed by replacement of bone with minimal physical changes in respond to different kinds of stress produced by physical activity or mechanical loads throughout a life span. The fundamental purpose of bone remodelling is to regulate calcium homeostasis, repair micro damaged bones and also to shape and sculpture the skeleton frame during growth.

The process of bone remodelling is activated through the formation of a bone-modeling unit which consists of three types of bone cells which are osteoblast, osteoclasts and osteocytes. Osteoclasts are responsible to break down old calcified bone in the process of bone resorption. They are activated by growth factors, cytokines and proteins present in the bone matrix. On the other hand, the function of osteoblasts is to produce bone matrix when mineral is added to form calcified bone. These bone cells are activated by growth factors such as insulin-like growth factors I and II released by both osteoclasts and/or osteocytes. Osteocytes are found within the confines of bone rather than on the surface and

they commence the process of bone calcification by modulating osteoblast differentiation from non-calcium depositing to calcium depositing cells through the released of growth factors such as insulin-like growth factor I and the tissue growth factor β (Liu and Webster, 2007).

2.2.1 Osteoblasts

Osteoblasts arise from osteoprogenitor cells located in the periosteum and the bone marrow. They can be found on the periosteal and endosteal surfaces of bone. The estimated size range of an osteoblast cell is from 10 to 50 μm in diameter. Osteoblasts are responsible for new bone formation. During osteogenesis, osteoblasts which are differentiated from osteoprogenitor cells, proliferate actively and express genes for Type I collagen, vitronectin and fibronectin. Extracellular matrix begins to develop and mature during the end of proliferation while osteoblasts start to differentiate to calcium depositing cells. The synthesis of osteopontin, collagenase (mRNA expression for proteins) and alkaline phosphatase activity increased rapidly. When the mineralization process begins, osteoblasts are formed and deposit bone sialoprotein, osteocalcin and other matrix protein. Osteocalcin interacts with HA to mediate coupling to bone resorption by osteoclasts and bone formation by osteoblasts and osteocytes (Liu and Webster, 2007).

2.2.2 Osteocytes

An osteocyte, a star-like shaped cell is derived from matured osteoblasts trapped in the mineralized bone matrix. It implements a smaller contribution to

new bone formation compared to osteoblast. Osteocytes which possess extensive long branches are networked to each other via long cytoplasmic extensions that occupy tiny canals called canaliculi, which are used for exchange of nutrients and waste. Osteocytes have the ability to control the vascular invasion and osteoclast recruitment during bone remodelling by secreting vascular induction factors in response to ischemic conditions. Osteocytes activate a specific gene for osteopontin- an important factor for mediating bone remodelling caused by mechanical stress (Roberts and Hartsfield, 2004). This mechanism controls the bone remodelling by mediating or balancing, either osteoblastic activity to form new bone or osteoclastic activity to dissolve old bone (Liu and Webster, 2007).

2.2.3 Osteoclasts

Osteoclasts are derived from preosteoclasts which circulate in the blood. These cells lie in the small cavity of the bone resorption regions called Howship's lacunae. Osteoclasts are responsible for removing bone tissues by removing its mineralized matrix. They come in rather large size which can be up to 100 μm in diameter. When osteoclasts are attached on the disrupted bone surfaces to dissolve bone, they form a ruffled cell membrane edges. This is done to increase the surface area. Then, they produce tartrate-resistant acid phosphatase which release hyrogen ions via the carbonic anhydrase system ($H_2O + CO_2 \rightarrow HCO_3^- + H^+$). The presence of hydrogen ions lower the pH of the environment, thus increasing the solubility of HA crystals and lastly the organic component of bone matrix are removed by acidic proteolytic digestion (Liu and Webster, 2007).

2.3 Osteoinduction and its importance for bone healing

Osteoinduction is a process whereby osteogenesis is initiated. This mechanism of bone formation is commonly observed during the bone healing process.

Bone and its surrounding osteogenic tissues contain a number of undifferentiated cells in addition to the differentiated bone cells such as osteoblasts, osteoclasts and osteocytes. These undifferentiated cells are recruited to form osteoprogenitor cells and they play a critical role during bone healing as well as during the process of osseointegration of an implant. An undifferentiated mesenchymal cell can be transformed into a preosteoblast, a process which constitutes bone induction, with the right stimulus such as the inductive agent. There are many studies in the past describing bone induction at various host sites and these authors have used gall bladder epithelium, alcohol extracts of bone and transplants to muscles or the anterior chamber of the eye, to demonstrate the formation of heterotopic bone. A widely acceptable method to demonstrate the osteoinductive property of a particular agent is to inject it into a heterotopic bed such as a muscle pouch and subsequently analyse for any potential bone formation. Inductive agents naturally function in bone surroundings too. However, it is a challenge to differentiate between bone induction and bone conduction in an orthotopic site (Alberktsson and Johansson, 2002).

Research into osteoinduction dates back to Urist's experiment in the mid 1960s (Urist, 1965). In this experiment, demineralised bone was used as an osteoinductive agent. Later, Urist *et al.* (1986) managed to isolate a soluble

glycoprotein called bone morphogenetic proteins (BMP) as the inductive agent. The BMP belong to the transforming growth factor (TGF)-β-family of growth factor. To date, a great number of research projects had arisen from there, involving various types of BMP. BMP are naturally released in response to trauma or at bone remodelling. So far, they are the only known inductive agents. Besides BMP, other factors such as physical stimuli for example, stress or types of electrical signals otherwise applied have been regarded as, directly or indirectly, influencing bone induction.

During, osteoinduction, preosteoblasts are formed by recruiting immature cells. This process is a basic biological mechanism that normally takes place in fracture healing and healing at the implant site. Although pre-existing osteoblasts (i.e. before injury) may assist to form new bone, these cells only contribute a minor portion of new bone needed in a fracture healing situation.

According to a study by Frost (1992), injury to the bone, marrow and soft tissue triggers the subsequent repair. The injury releases a wide range of local, biochemical and biophysical mediators that stimulate these pre-osteoblastic cells. These mediators also play a role in the cellular differentiation and organisation of these cells, while others may provide mitogens. Osteoinduction starts immediately after the injury and this process would be very active during the first week thereafter, even though the action of the newly recruited preosteoblasts is not obvious until several weeks later, in the callus stage. However, it is a challenge to differentiate between bone induction and bone conduction in an orthotopic site (Alberktsson and Johansson, 2002).

2.4 Anatomy of rabbit teeth

Rabbits are lagomorphs with two pairs of upper incisors. The second pair of incisors which is often regarded as the peg teeth is small and is located immediately behind the larger incisors. The lower jaw of the rabbit is called the mandible while the upper jaw is called the maxilla. The mandible is narrower than the maxilla. The deciduous teeth of a rabbit are rarely seen because they are shed before birth or shortly after birth. Besides that, rabbits have a set of cheek teeth located in the back of the mouth which are used for grinding. The cheek teeth are divided into upper molars and lower molars. The upper molars consist of six teeth with three premolars and three molars while the lower molars consist of five teeth with two premolars and three molars on each side (refer Figure 3.3). The physical structure of a rabbit's teeth is accommodated for the ingestion of fibrous diet. The incisors and the cheek teeth are open rooted and they grow continuously at a fast rate. The growth of the incisor has been measured at 2.0 to 2.4 mm per week. The rate of growth is dependent on the rate of eruption and the rate of attrition (Hartcourt-Brown, 2007).

Rabbit's teeth are cylindrical in shape. Enamel and dentine are formed continuously from germinal tissue at the extremity of the tooth that is buried in the jaw. Hence, the buried section of the tooth is eventually supragingivally exposed. In rabbits, enamel coats both the exposed and buried parts of the teeth and for this reason; there is no distinguishable root, crown, or neck. Therefore, terms such as *anatomical crown* is used to describe the whole tooth, *exposed* or *clinical crown* and *reserve* or *submerged crown* or *clinical root* is used to describe the

supragingival and subgingival sections. On the other hand, the terms *apex*, *apical*, and *periapical* refer to the extremity of the tooth that is embedded in the jaw (Hartcourt-Brown, 2007).

The wear against opposing teeth during mastication and chewing movements that rabbits make during rest help to maintain the shape of the occlusal surfaces of the teeth. Mastication and the occlusal relationship of the teeth in rabbits has been the subject of a study as early as 1958, using cinematoradiography to investigate the relative position and movements of the jaws. Weijs and Dantuma (1981) have reported that the movement of the rabbit's jaws depends on the type of food that is eaten. A crushing action was used when carrots were eaten while a shearing action was used when hay was eaten. Both actions of crushing and shearing were used when pellets were eaten. These actions of the jaws are normal and enable rabbits to eat a wide range of food. The sharp enamel ridges on the cheek teeth play an important role in mastication, particularly when fibrous material is consumed and needs to be reduced to small particles before it is swallowed. The following clinical findings show that a rabbit has a good dentition (Harcourt-Brown, 2007):

- Rabbits with healthy appetite and will easily eat a large amount of hay.
- Rabbits with healthy coat. Rabbits usually use their incisors to remove dead hair and parasites from their coat and use their tongues to lick and clean the fur. Dental disease is a main differential diagnosis for a rabbit with unhealthy fur or heavy ectoparasite burdens.
- Palpably smooth, straight ventral borders to the mandible.
- Short, smooth, well-enameled incisors that occlude ordinarily.

- Enamel ridges that form a series of upright points along the lingual edge of the row of cheek teeth. The ridges can be observed during oral examination with an otoscope.

2.5 Tissue engineering

Tissue engineering is the state of the art in applied biomedical research aimed at developing procedures and new biomaterials for the fabrication of new tissues. These new tissues are then introduced into a patient to promote, repair or replace existing tissues in the body. Tissue engineering is based on principles of cell biology, developmental biology, and biomaterials science (Slavkin and Bartold, 2006).

This approach was contrived because of constant lack of donor organs and tissues. It has also been expanded to rectify damaged tissues and develop newly improved methods in surgical procedures (Williams *et al.*, 1999).

The recent progress in growth factor biology and biodegradable polymer constructs have been the focus for successful tissue engineering of cartilage, bone, and related tissues (Slavkin and Bartold, 2006).

There is a need to exert mutual influence between three components in the implementation of successful tissue engineering (Slavkin and Bartold, 2006):

- The implanted and cultured cells that will create the new tissue.
- A biomaterial to represent a scaffold or matrix to hold the cells.
- Biological signalling molecules that act as a guide for the cells to form the desired tissue type.

Generally, there are three well-explained approaches currently being used to engineer new tissue (Williams *et al.*, 1999). They are:

- Infusion of isolated cells or cell substitutes
- Use of tissue-inducing materials
- Implantation of cells seeded in scaffolds

2.6 Guided bone regeneration

Guided bone regeneration is a specialized sub-area of tissue engineering. In this technique, the bony tissue regenerates and grows on or along the surface of host tissue guided by the structure of an implanted scaffold. The emphasis of new bone growth on or along the surface of the host can be gained by a variety of materials used as scaffolds, for example bioceramics or by the porosity of an implant. The criteria in selecting an appropriate scaffold implant i.e. its physical appearance and its fabrication, will depend on the application and implantation site. Scaffolds can be in various forms such as membranes, meshes, plates, screws, plugs, or rods.

Some active material such as ceramics, ceramic-glasses and bioactive glasses are known to have osteoconductive properties. Hence, they are suitable for guided regeneration of bone tissue, for example in filling bone defects. They can also be manufactured in a different form, for example, porous form which seems to improve bone ingrowth and bonding. Bioceramics and glasses vary from biologically stable to bioactive and even bioresorbable. These materials are brittle,

therefore it limits their applicability. Polymers are more rigid and more flexible compared to ceramics. The properties of polymers can be varied to a great extend by modifying the structure of the polymer. Manufacture of composites of polymers and ceramics has been attempted because of the combination of osteoconductivity and stiffness of polymer to further discover a more applicable material for regenerating bone (Kellomaki *et al.*, 2000).

2.7 Bone substitutes

Beside blood, bone graft is the second most common transplantation tissue. Bone-grafting procedures are widely used for the repair of bone defects in orthopaedics, neurosurgery and dentistry. Bone grafts have the ability to promote tissue or bone regeneration through several mechanisms. The aim of these procedures is to stimulate bone-healing. Other clinical applications of bone grafts include spinal fusions, filling of bone defects after tumour removal and bone augmentation procedures in congenital diseases. In general, grafts can be divided into four categories which are autograft, allograft, xenograft and alloplast (Giannoudis *et al*., 2005).

2.8 Bone graft characteristics

2.8.1 Osteogenesis, osteoinduction and osteoconduction

Osteogenesis, osteoinduction and osteoconduction are three important properties of bone regeneration in which a bone graft may possess. Osseointegration would be the ultimate bonding between the host bones and grafting material. During transplantation, osteoprogenitor cells residing within the donor graft, may survive and these cells have the tendency to proliferate as well as differentiate to osteoblasts and eventually osteocytes. These cells represent the osteogenic potential of the graft. Hence, osteogenesis is the process of new bone formation by cells from the host itself or the graft.

Osteoinduction is the process of stimulation and activation of host mesenchymal stem cell from the surrounding tissue or mesenchymal stem cells from the graft, which subsequently differentiate into chondroblasts or osteoblasts. During this process, a cascade of signals is mediated along with the activation of several extra- and intracellular receptors. These receptors are the most important and they belong to the TGF-beta super-family.

Osteoconductive graft materials may merely act as a three dimensional scaffold on which host bone might grow. Osteoconduction involves the facilitation and orientation of blood-vessel and the creation of new Haversian systems into the bone scaffold. This is typically followed by osseointegration, a process which describes the surface bonding between the host bone and the grafting material (Giannoudis *et al*., 2005).

These bone graft characteristics can be found in various types of bone graft substitutes i.e autograft, allograft, xenograft and alloplast. However, the types of bone graft substitutes may or may not have all these characteristics mentioned earlier on (Table 2.1).

2.9 Types of bone graft substitutes

2.9.1 Autograft

An autograft is the tissue removed from one part of the skeleton and transferred to a different part of the same patient. In oral surgery and implant dentistry, autologous bone is the gold standard of bone grafting because it provides optimal osteoconductive, osteoinductive, and osteogenic properties needed for bone regeneration. There is also no immune reaction and the microscopic architecture is perfectly matched. However, there are several disadvantages from harvesting the autologous bone, as it lengthens the overall surgical procedure and is usually followed by complication of residual pain.

For most grafting purposes required with dental implants, the grafts are usually obtained intraorally, for example, from the extraction sockets, edentulous ridges, ramus, symphysis, tuberosity or the surrounding buccal plate. This purpose allows the surgery to stay within the mouth. Alternatively, when there is a shortage of bone available in the mouth or when larger grafts are required, bone will be transferred extraorally from other parts such as the iliac crest or tibia (Bashutski and Wang, 2009).

2.9.2 Allograft

An allograft is defined as a bone graft between two individuals of the same species but is genetically dissimilar. Allograft is the most frequently chosen bone substitute and it is always the surgeon's second option because of its high success rate. In addition, no additional surgical procedure is required to obtain bone from a donor site. However, the disadvantages would be the potential to transfer viral disease, cost and graft contamination during processing.

Allografts can be separated into cortical or cancellous component and can be prepared either in fresh, frozen or freeze-dried form. Fresh allografts are hardly used as they might kindle an immune response or even transmit diseases. The commonly used allografts are freeze-dried bone allografts and decalcified freeze-dried bone allografts. Decalcifying the bone allografts exposes the bone morphogenetic protein which has osteoinductive properties but this type of graft has a higher resorption rate compared to the freeze-dried bone allografts and thus has the tendency to act as a less effective scaffold (Bashutski and Wang, 2009).

The concern regarding viral disease transmission is practically eliminated through tissue processing and sterilisation. Nevertheless, both freezing and irradiation that eliminate the cellular phase of the allograft modify the processes of graft incorporation and affect its structural strength. Hence, allografts might be osteoconductive or osteoinductive, depending on the processing method used. Frozen and freeze-dried allografts are more osteoconductive but are considered to

have a weaker osteoinductive capabilities compared to fresh allograft (Giannoudis *et al*., 2005).

2.9.3 Xenograft

Xenograft is defined as a tissue graft taken from one species and transfer into a different species. Bovine and porcine are common animal sources for intraoral bone graft substitutes. This bone has a similar microscopic structure as human bone which stimulates bone growth. All organic constituents in this grafting material are removed during processing whereby antigenicity is no longer a concern. Nonetheless, xenografts are considered as osteoconductive materials.

Immune rejection remains as the biggest challenge for this type of graft and the risk of transmission of animal viruses to patients have yet to be resolved. In addition, tissue or bone regeneration with xenografts might be uncertain (Bashutski and Wang, 2009).

2.9.4 Alloplast

An alloplast is a synthetically derived bone graft material not coming from an animal or human origin. These synthetic bone grafts possess only two characteristics of an ideal bone graft material which is osseointegration and osteoconduction. As a bone graft, this material should be biocompatible, show minimal fibrotic reaction, undergo remodelling and support new bone formation as

far as possible. Alloplast can be divided into hydroxyapatite, beta-tricalcium phosphate, non-ceramic, polymer or bioactive glass.

Nevertheless, these materials demonstrated several disadvantages in clinical settings such as low or unpredictable resoprtion, difficulty in handling, and poor clinical results with inflammatory foreign body reaction occasionally (Giannoudis *et al*., 2005).

Table 2.1 Characteristic of available bone graft materials and their sources (*adapted and modified from* Bashutski and Wang, 2009).

	Types of bone graft			
	Autograft	**Allograft**	**Xenograft**	**Alloplast**
Source	Host	Different individuals	Different species	Synthetic
Properties	Osteogenic Osteoinductive Osteoconductive	Osteoinductive Osteoconductive	Some osteoinductive Osteoconductive	Osteoconductive

2.9.4.1 Polymers

Polymers can be either in synthetic or natural forms. They are the largest diverse class of biomaterials. Polymeric biomaterials are vastly used in several

applications, particularly medical and pharmaceutical. Their contribution to the quality and effectiveness of health care is greatly significant.

Like other materials, polymers can be available in a wide variety of compositions and properties. They can be produced and then processed to form complex shapes with different sizes according to the requirement of its final application. Besides that, their surface properties and fabrication can be modified by physical, chemical or biochemical means. This modification is important in biological applications. However, the main disadvantage is that some portion of the modified material may leach out during use, and this may cause undesirable effects in the host (Piskin, 2002).

2.10 Biodegradable polymers

2.10.1 Overview

There is a great number of biodegradable polymers which can be synthesized by microorganisms using renewable resources. At the same time, microorganisms and enzymes that are capable of degrading these polymers have been identified. Biodegradable plastics have been developed as surgical implants in vascular and orthopaedic surgery as implantable matrices for the controlled long-term release of drugs inside the body, as absorbable surgical sutures, and for use in the eye (Chandra and Rustgi, 1998).

One definition of biomaterial is as a nonviable material used in medical device applications that is intended to interact with a biological system (Chandra

and Rustgi, 1998). The term biocompatibility (which deals with the tissue reaction to foreign materials), is defined as the ability of a particular material to perform with an appropriate host response in a specific application. Biomaterials are used for the following purposes:

(a) To replace tissues that are diseased or damaged such as in joint replacements, artificial heart valves and arteries, tooth reconstruction and intraocular lenses.

(b) To aid in repairing tissues in the form of sutures and also bone fracture plates, ligament and tendon repair devices.

(c) To substitute the entire or part of the function of the major organs, such as in haemodialysis (replacing the function of the kidney), oxygenation (lungs), left ventricular or whole heart assistance (heart), perfusion (liver), and insulin delivery (pancreas).

(d) To distribute drugs to the body via targeted sites for example, either directly to a tumour or sustained delivery rates (insulin, pilocarpin, contraceptives).

Plastics have become an essential and irreplaceable part of twentieth century life. It is difficult to degrade petrochemical plastics and because of that, concern over the management of this material particularly disposal of plastics has led to a general consensus to reduce, reuse and where possible, recycle plastic materials. The bacterially derived polyhydroxyalkanoate (PHA) polymers provide an alternative to petrochemical plastics because they have similar material properties but are naturally produced and are therefore biodegradable (Hammond and Liggat, 1995).

The production of plastic polymers from renewable resources has gradually increased in interest and is constantly expanding because it is understood and apparent that fossil fuels and petrochemicals are a finite resource. Furthermore, the accumulation of synthetic non-degradable plastics in the environment and their combustion products are serious pollution problems.

With the persistence of plastics in the environment, there has been a worldwide research effort to develop biodegradable polymers to overcome environmental pollution due to petrochemical plastics (Swift, 1992).

Degradable polymers have found applications in a lot of areas, for example, in medicine such as in sutures, wound dressings, surgical implants, and controlled-release drug delivery systems. Besides that, there is a great demand for degradable plastic films for use in garbage bags, food and beverage containers, and for mulching. Several industries are currently developing biodegradable polymers for ultimate use in the consumer products. Three major requirements are established in order to get general acceptance of biodegradable polymers in the environment (Swift, 1992);

a) to rationalize the usage of these materials

b) a comprehensive explanation which exhibit that what these materials do in the environment is acceptable to everyone

c) to implement test protocols to verify that these materials do as expected in the environment

Degradable polymers/plastics play important roles in conserving the natural ecosystems i.e. to reduce the pollution and to maintain a proper balance in the environment.

2.10.2 Biodegradable polymer as a substitute for plastic

Biodegradable polymer is defined as a polymer in which the degradation is mediated at least partially by a biological system (Li and Vert, 2002).

Biodegradable macromolecules can be processed specially to cater for controlled degradation under the inherent environmental stress in biological systems either unaided or by enzyme-assisted mechanisms. Progressively, it was realized that biodegradable systems could be of great relevance for temporary therapeutic applications in surgery, in pharmacology and also in tissue engineering. The first biodegradable synthetic polymer was poly(glycolic acid) which was invented in 1954. Unfortunately, this polymer was rejected because of its poor thermal and hydrolytic stabilities which were not suitable to be used as a conventional plastic material. Twenty years later, it became the first biodegradable suture material not related to natural polymers. From there, many studies of the mechanisms of biodegradation of synthetic polymers were motivated. Medical applications of biodegradable polymers have led to vital developments, such as the controlled release of drugs, fertilizers and pesticides, resorbable surgical implants, skin grafts and bone plates (Huang, 2002).

2.11 Polyhydroxyalkanoates (PHA)

Organisms are able to accumulate large amounts of carbon and energy in the form of osmotically inert polymers. These polymers include polysaccharides such as glycogen in bacteria and animals, and starch in algae and higher plants. Beside polysaccharides, other highly interesting natural storage compounds like polyesters can be formed by bacteria (Anderson and Dawes, 1990).

Polyhydroxyalkanoates are a group of natural polyesters that are produced by bacteria in the presence of excess carbon (Anderson and Dawes, 1990). They are one of the most fascinating and largest groups of thermoplastic polymers known, with over 100 different types currently produced from a variety of different monomer types as shown in Figure 2.1. Their properties span a wide range, including materials that resemble polypropylene and others that are elastomeric. They possess different chemical properties, either from the length of the pendant groups which extend from the polymer backbones, or from the distance between the ester linkages in the polymer backbones. The monomeric composition of PHA depends on the bacterial strain and on the carbon source used, for example glucose, during the accumulation phase (Williams *et al.*, 1999).

PHA can be classified as short-chain-length PHA(scl-PHA) which consists of 3 to 5 carbon atoms in the repeating hydroxyalkanoate units, medium-chain-length PHA (mcl-PHA) which have 6 to 15 carbon atoms in the repeating hydroxyalkanoate units, and long-chain-length PHA (lcl-PHA), which have more than 15 carbon atoms in the repeating hydroxyalkanoate units (Jendrossek *et al.*, 1996).

Generally, PHAs with short pendant groups are hard crystalline materials, whereas PHAs with longer pendant groups are elastomeric. PHAs offer a wide range of mechanical properties and they are also biodegradable. They are broken down in soil and water by bacteria and fungi in the environment, in the similar way that plant and animal waste is degraded. Their biodegradability and the fact that they can be produced from renewable resources make PHA of considerable commercial interest for use in various industrial applications (Anderson and Wynn, 2001).

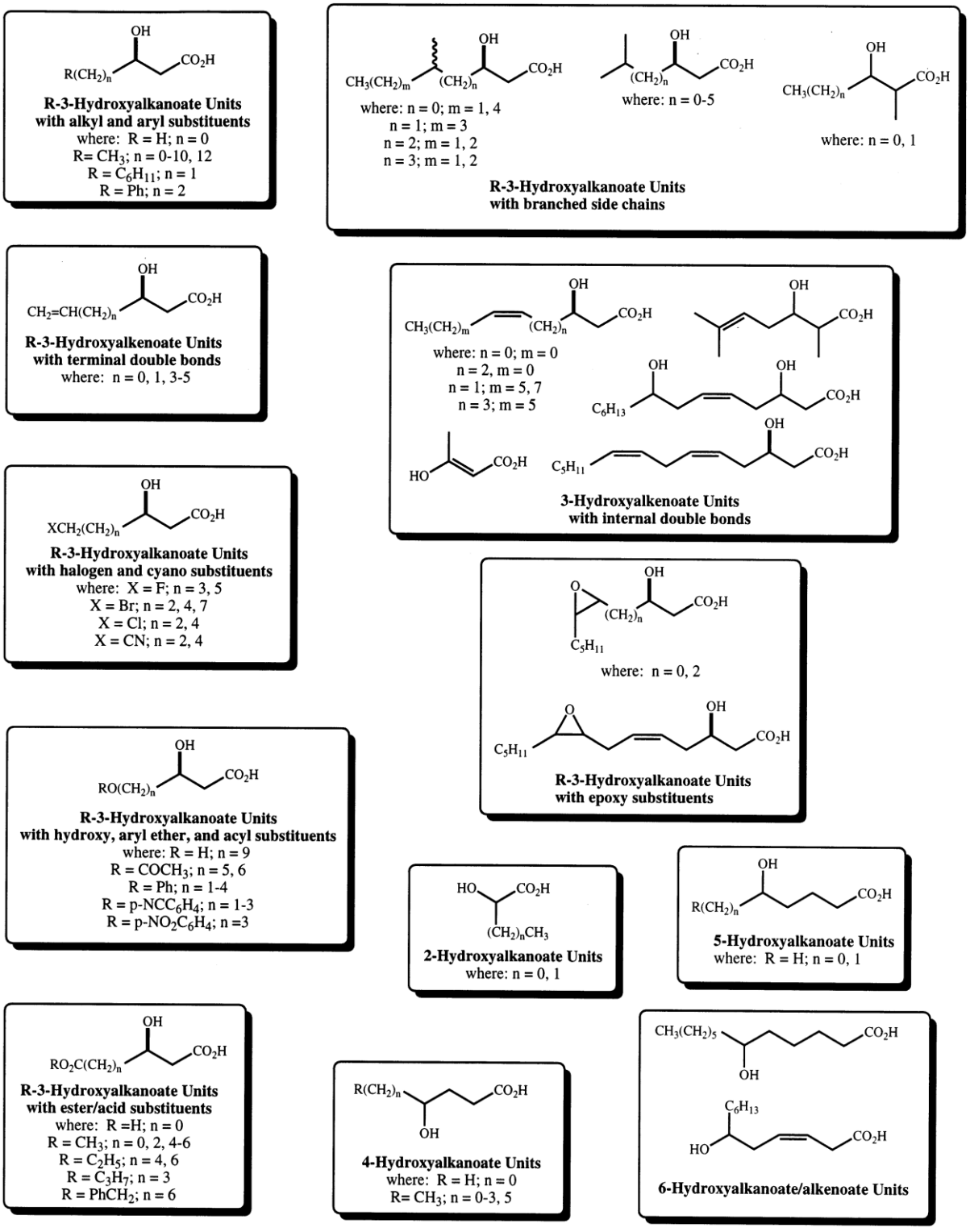

Figure 2.1 Examples of monomers that can be incorporated into PHA polymers (*adapted from* Williams *et al.*, 1999)

2.11.1 PHA composition and properties

PHAs are linear polyesters which made up of hydroxyacid monomers. Most common are 3-hydroxyacid monomers and 3-hydroxyacids with carbon chain lengths from C_3-C_{14} that have been identified in the range of PHA produced by bacteria. Most PHA comprise two or more different monomers and is known as heteropolymers except PHB which is a homopolymer. Generally, the polymers are composed of a random sequence of the constituent monomers rather than having different monomers in separate chains. In addition to 3-hydroxyacids, various other hydroxyacid monomers have been identified in PHA. The composition of PHA depends on the organism as well as the carbon sources available during polymer accumulation and research has shown that, collectively, bacteria are able to incorporate over a hundred different hydroxyacid monomers in PHA (Anderson and Wynn, 2001). Several examples of bacterial PHA are given in Table 2.2

PHA monomers are produced from 'precursor substrates' that have a related chemical structure. For example, *Pseudomonas oleovarans* synthesises PHA containing mainly 3-hydroxyoctanoate monomers from octanoic acid or octane, and *Ralstonia eutropha* produces 4-hydroxybutyrate and 3-hydroxypropionate monomers from 4-hydroxybutyric acid and 3-hydroxypropionic acid, respectively (Anderson and Wynn, 2001).

The physical properties of PHA are dependent on its constituent monomers. For example, PHB is hard and inflexible, but polymers with longer side chains are soft elastomers. In some cases, it is easy to control the composition of PHA.

Therefore its physical properties can easily be controlled by altering the composition of the medium. For example, *Ralstonia eutropha* produces PHB from glucose and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHB/V) from glucose plus propionic acid. PHB/V is a copolymer made up of 3HB and 3HV monomers, and its composition can be controlled by varying the concentrations of glucose and propionic acid in the medium during the polymer accumulation phase. PHB is hard and brittle, but with the incorporation of a small proportion of 3-hydroxyvalerate (3HV) monomers into the polymer chain, will result in a stronger and more flexible plastic. This is investigated and applied in the commercial production of PHB/V (Anderson and Wynn, 2001).

In some cases, bacteria can synthesize PHA monomers that have no relation to the structure of the carbon sources provided. For example, fluorescent pseudomonads produce PHA containing 3-hydroxydecanoate from many carbon sources and some *Rhodococcus* and *Nocardia* species produce PHB/V containing a high proportion of 3HV monomers, from a variety of carbon sources (Anderson and Wynn, 2001).

Tan *et al.* (1997) used saponified palm kernel oil (SPKO) as carbon substrate to synthesize PHA from *Pseudomonas putida*. The polyester produced has similar properties to the derivatives of lauric and myristic fatty acid which is soft, flexible and transparent in presence. The synthesized PHA consist of n-alkanoate monomers ranging from C_6 -C_{14} with C_8 as the main component of the material.

Table 2.2 Composition of PHA produced from various carbon sources by a range of bacteria (*adapted from* Anderson and Wynn, 2001)

Organism	Carbon source (s)	Composition of PHA										
		3-hydroxyacid monomers										Other monomers
		C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	C_{11}	C_{12}	
Ralstonia eutropha	Glucose	×										
R. eutropha	Glucose + propionic acid	×	✓									
R. eutropha	Glucose + 4-hydroxybutyric acid	×										✓ (4HB)
Comamonas acidovorans	Glucose + 4-hydroxybutyric acid	✓										× (4HB)
Alcaligenes latus	Sucrose + 3-hydroxypropionic acid	✓	×									
Pseudomonas oleovorans	Octanoic acid				✓		×		✓			
P. oloevorans	Nonanoic acid				✓	✓	✓	×	✓	✓		
P. aeruginosa	Gluconic acid				✓		✓		×		✓	
Rhodococcus ruber	Glucose	✓	×									
× principal monomer present in PHA												
✓ other monomers – 4HB 4-hydroxybutyrate												

2.11.2 PHA biosynthetic pathway

Several different metabolic pathways for the synthesis of PHAs are known to occur in bacteria. There are four different pathways which have been identified for the synthesis and incorporation of almost all the known constituents of bacterial PHA (Steinbuchel, 1992).

First, acetyl coenzyme A (acetyl-CoA) is converted to P(3HB) via three enzymatic steps in *A. eutrophus*. A biosynthetic β-ketothiolase catalyzes the formation of a carbon-carbon bond of two acetyl-CoA moieties. An NADPH-dependent acetoacetyl-CoA reductase catalyzes the reduction of acetoacetyl-CoA formed in the first reaction to D-(-)-3-hydroxybutyryl-CoA. The third reaction of this pathway is catalyzed by the PHA synthase, which links the D-(-)-3-hydroxybutyryl-moeity to an existing polymer molecule by an ester bond.

The second type is the five-step PHA biosynthetic pathway in a photosynthetic bacterium, *Rhodospirillim rubrum*. Acetoacetyl-CoA formed by the β-ketothiolase is reduced by an NADH-dependent reductase to L-(+)-3-hydroxybutyryl-CoA. The latter is then converted to the D-(-)-3-hydroxybutyrul-CoA by two enoyl-CoA hydratases. The last step of this pathway is again the polymerization catalyzed by the PHA synthase.

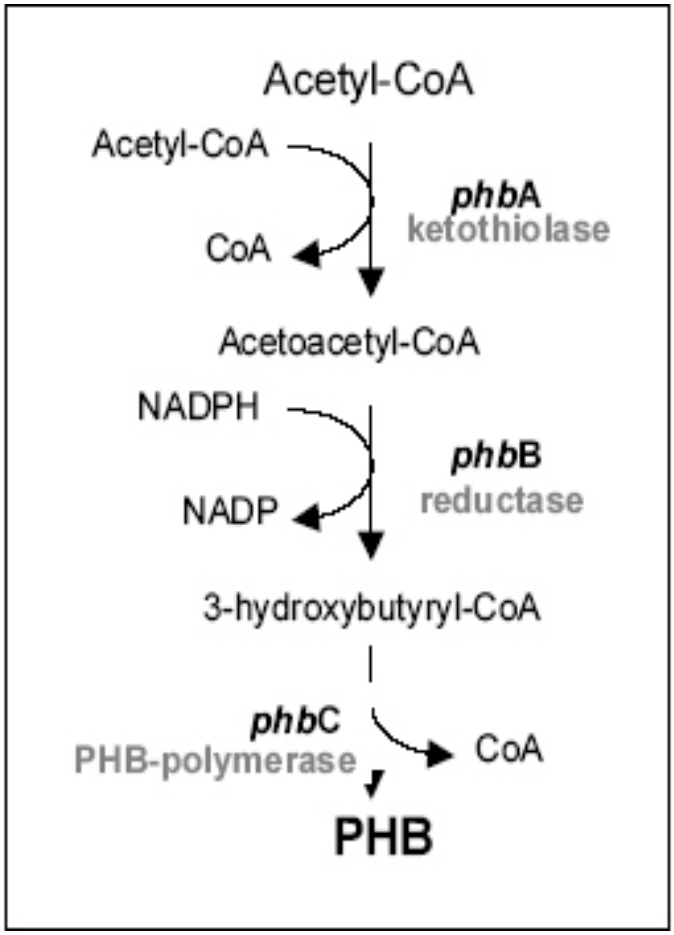

Figure 2.2 Metabolic pathway for the production of short-chain-length PHA in *Ralstonia eutropha* (*adapted from* Romano, 2002)

The third type of PHA-biosynthetic pathway seems to be active in most pseudomonads belonging to the ribosomal RNA homology group I. In this pathway, the intermediates from the β-oxidation of the activated fatty acids derived from alkanes, alkanols, or alkanoates are directed to PHA biosynthesis. For example, *Pseudomonas oleovorans* accumulates polyester that contains 3-hydroxyactanoate and 3-hydroxyhexanoate as the main constituents when cultivated on octane, octanol or octanoate.

The fourth type of pathway is found in almost all pseudomonads belonging to the rRNA homology group I, except *P. oleovorans*. Copolymers consisting of medium-chain-length 3-hydroxyalkanoates are synthesized from acetyl-CoA. The precursors for PHA synthesis are derived from de novo fatty acid synthetic pathways. The biosynthesis of PHA mainly consisting of 3-hydroxydecanoate in *Pseudomonas aeruginosa* follows this pathway (Lee and Choi, 1999).

Figure 2.2 exhibit a typical example of biosynthetic pathway for the production of scl-PHA in *Ralstonia eutropha* (Romano 2002).

2.11.3 Biodegradation of PHA

Several parameters that can influence the rate of biodegradation of PHA are the type of environment, microbial population, the availability of water or moisture, temperature, the shape and thickness of the plastic material, surface texture, porosity and crystallinity, and the presence of other components in the plastic, such as fillers or colouring agents.

Methods that determine the degradability of plastic materials are usually based in direct visual observation of plastic material, quantitative determination of microbial growth on plastic material (measured by turbidity or protein and phospholipids content), polymer utilization by micro organisms (by gravimetric or visual observation), determination of changes in polymer characteristics during incubation (by tensile strength or molecular weight), and determination of microbial activities such as titrimetric, electrochemical, or monometric activities.

Field or *in situ* experiments in which the samples are placed in the desired environments are required for the determination of biodegradability; because these laboratory test methods (as written above) are difficult to employ in the natural environment (Lee and Choi, 1999).

In aerobic conditions, PHA is degraded into carbon dioxide and water while in anaerobic conditions; it is finally degraded into methane, water and carbon dioxide. Degradation of P(3HB) occurs in to steps. First, P(3HB) is degraded into 3HB oligomers, preferentially 3HB dimmers, by the extracellular P(3HB) depolymerase. Secondly, an oligomer hydrolase cleaves the dimmer into 3HB monomer. A number of aerobic and anaerobic PHA-degrading bacteria and fungi have been isolated, and the extracellular PHA depolymerises from several bacteria have been cloned and characterized (Lee, 1996).

Ho *et al.* (2002) reported that mcl-PHA was degraded in tropical river water with a weight loss of 71.3 % of the PHA film in 86 days. Shorter-chain monomers in mcl-PHA were selectively removed from the PHA molecule which suggested enzymatic action. On the other hand, Lim *et al.* (2005) reported the biodegradation of similar mcl-PHA film in tropical forest and mangrove soils. There was weight loss in the test material and the degradation was by surface erosion. However, the integral properties of the films were maintained.

2.11.4 Applications and commercial aspects of PHAs

PHAs have been drawing considerable industrial interest as candidates for the biodegradable and biocompatible plastics and elastomers for a wide range of applications, including packaging, controlled drug release and biomedical materials (Lee and Choi, 1999).

Besides that, mcl-PHAs can also be used in a number of applications, for example, elastomeric (rubber-like) materials, cheese coating, adhesives, coatings and paint binders. The massive exploitation of PHAs in tissue engineering has been reported in several medical applications; or as stocks for the stereoscopic-drug industry (Romano, 2002).

The bacterium *Ralstonia eutropha* accumulates scl-PHA, which can be processed and applied in a way similar to commodity plastics. The heteropolymer PHB / HV is commercially known as Biopol^{TM}. Biopol^{TM} can be processed on most types of conventional thermoplastic melt-processing equipment. Examples of product forms include a wide range of injection-moulded products and extrusion blow-moulded bottles. Foamed products can also be produced. In addition to the properties and processes associated with conventional thermoplastics, the Biopol^{TM} resins offer additional features of naturalness and biodegradability. The fermentation production process and the use of renewable feedstock clearly differentiate these products from conventional petrochemically derived plastics. Biodegradability expands the range of disposal options. In addition to conventional waste management routes such as recycling and incineration, these materials have benefits in products which may be disposed in

sewage systems, the soil or managed landfill sites. The disposable hygiene and agricultural markets fit into this category.

On the other hand, medical applications can exploit the key features of biocompatibility and relatively slow *in vivo* biodegradation reported for Biopol™ polymers. There is evidence in experiments suggesting that Biopol™ resins are extremely well tolerated when implanted within the body. The Biopol™ implant, however, was completely biodegraded and the resulting repaired pericardium tissue gave far fewer postoperative adhesions compared to control experiments (Hammond and Liggat, 1995).

2.11.5 Applications of PHAs in medicine

At present, PHA and its composites are thought to have high potentials as apparent materials for medical devices such as sutures, bone plates, surgical mesh and cardiovascular patches and many more (Table 2.3). A recent major advancement for this group of biomaterial is the clearance acquired from the Food and Drug Administration of the United States of America for the use of P(4HB)-derived TephaFLEX® as absorbable sutures (Ying *et al.*, 2008).

Table 2.3 Potential applications of PHA in medicine (*adapted from* Zinn *et al.*, 2001).

Type of application	Products
Wound management	Sutures, skin substitutes, nerve cuffs, surgical meshes, staples, swabs
Vascular system	Heart valves, cardiovascular fabrics, pericardial patches, vascular grafts
Orthopaedy	Scaffolds for cartilage engineering, spinal cages, bone graft substitutes, meniscus regeneration, internal fixation devices (e.g., screws)
Drug delivery	Micro- and nanospheres for anticancer therapy
Urology	Urological stents
Dental	Barrier material for guided tissue regeneration in periodontitis
Computer assisted tomography and ultrasound imaging	Contrast agents

Because they are bioabsorbable and biocompatible, PHAs are particularly interesting. The metabolism and excretion of some monomers incorporated into PHA are well understood. For example, the monomeric component of P(3HB), (R)-3-hydroxybutanoic acid (3HB), is a ketone body which exist at concentrations of 3 - 10 mg per 100 ml blood in healthy adults (Williams and Martin, 2002). The monomeric component of P(4HB), 4-hydroxybutanoic acid (4HB), can also be located in various organs and it is widely distributed in the brain, kidney, heart, liver, lung and muscle of the mammalian body (Nelson *et al.*, 1981). Moreover,

the hydroxyl acids liberated during PHA *in vivo* breakdown are reported to be considerably less acidic and less inflammatory compared to many other synthetic absorbable polymers such as poly(lactic acid) (PLA) (Taylor *et al.*, 1994). Nevertheless, P(3HB) has limited application due to its properties such as high brittleness, poor process ability and slow degradation. Therefore, researchers in this field of interest had shown great progress over the past 20 years and it is now possible to design and synthesize various kinds of PHA to further improve the inferior properties of P(3HB) (Ying *et al.*, 2008).

Quite a number of applications were proposed, tested and then applied in medicine. For example, PHBV is non-toxic and compatible with living cells, producing an extremely moderate foreign body response and the biodegradation rate *in vivo* is low. The main degradation product for PHBV is R-β-hydroxy-butyric acid, which is typical metabolite that can be found in mammalian cells. Controlled drug release microcapsules, surgical sutures, surgical swabs, wound dressings, lubricating powder for surgeon's gloves and even blood-compatible membranes are common applications that can be quoted for consideration in hospitals. One of the advantages of using PHB or PHBV is that unlike cotton, small pieces of these materials such as from swab or dressing can be left in the wound without the risk of inflammation (Chodak, 2002).

2.11.5.1 PHA as drug carrier

PHAs have been used as drug carriers due to their biodegradability and biocompatibility as early as in the 1990s (Pouton and Akhtar, 1996). For example, microspheres of PHB loaded with rifampicin were investigated for their use as a chemoembolizing agent (Kassab *et al.*, 1997). It was reported in this study that the drug release of all microspheres was very fast. Almost 90 % of the drug was released within 24 hours. Hence, it was possible to control the drug release rate by the drug loading and the particle size (Zinn *et al.*, 2001).

Also the usage of PHA as an antibiotic-loaded carrier to treat implant-related and chronic osteomyelitis was investigated by Yagmurlu *et al*. (1999). In this study, sulbactamcefoperazone, a type of antibiotic, was integrated into rods ($1 \times 0.3 \times 0.3$ cm^3; 100 mg) made of poly(3-hydroxybutyrate-*co*–22 mol%-3-hydroxyvalerate) and implanted into a rabbit's tibia that was artificially infected by *S. aureus*. After fifteen days, the infection subsided and almost complete healing was found on the thirtieth day.

All the mentioned examples show that the release of drugs from microspheres made of scl-PHA occurs at excessive rates. One of the reasons of this effect may be due to the manufacturing process, for example, the level of porosity. However, it can be hypothesize about that PHAs with a lower crystallinity, for example mcl-PHA would be a better candidate used for granules with lower drug release rates. Yet, because of the low availability of mcl-PHA, such research is still limited (Zinn *et al.*, 2001).

2.11.5.2 PHA as scaffold material in tissue engineering

A study done by Williams *et al.* (1999) has defined five parameters that scaffolds need to fulfil for successful tissue engineering: They are:

(1) biocompatibility

(2) support for cell growth and cell adhesion

(3) guide and organize the cells

(4) allow ingrowth of cells and passage of nutrients and waste products,

(5) biodegradable without forming toxic compounds

In tissue engineering, PHA scaffolds can be implanted together with the supporting scaffold. This approach was exemplified by Sodian *et al.* (2000) that used scl-PHA for the fabrication of a tri-leaflet heart valve scaffold.

DegraPol, a block-copolyesterurethane, chemically synthesized from PHB-diol and α,ω-dihydroxy- poly(Є-caprolactone-block-diethylene-glycol-block-Є-caprolactone), showed a good biocompatibility *in vivo* and *in vitro* (Saad *et al.*, 1999). The results in this study reported that the degradation products of a DegraPol foam are well phagocytized by macrophages and osteoblasts. However, at high concentrations of low molecular weight PHB (M_n ~ 2300) cell viability of macrophages and to a lesser extent of osteoblasts was reduced (Saad *et al.*, 1999). This study suggested that DegraPol is useful in bone healing processes, for example, in autologous osteoblast or chondrozyte transplantation (Zinn *et al.*, 2001).

2.11.6 Biocompatibility of PHAs

The definition of biocompatibility is substantial and it covers all the responses ranging from the biological system to the biomaterial. If a certain material is not biocompatible, it may be rejected at once or may cause other important problems, such as emboli, tissue necrosis or even tumour formation.

The implant of materials in tissues always generates a response. The main tissue response in the extravascular system is the inflammatory process, which starts off as a local reaction to injury, insult or infection. Inflammation may be induced biologically, chemically or physically. This complex process involves many proteins and cells. Indirectly, the chemical characteristics of a biomaterial, in the long-term, the released substances and the biodegradation products, may be responsible for foreign body reactions. If the foreign body reaction in soft tissue to a biomaterial is a mild inflammatory response, healing may occur quickly, and eventually, the implant will perform effectively (Piskin, 2002).

In addition, cell attachment, spreading, and growth are important criteria to evaluate the biocompatibility of a biomaterial. These processes are influenced by certain factors i.e. the various types of surface composition, surface free energy and morphology. The process of cell attachment normally takes place in two steps. Initially, the random cell adhesion would occur on the surface of biomaterial, which is mainly governed by physical and chemical interaction between the biomaterial and cells. This is followed by specific cell adhesion on the surface (Li *et al*., 2005).

If a medical application is being considered, it is of no uncertainty that the biological response to PHA polymers *in vivo* is the most important property of these biomaterials (Williams and Martin, 2002).

In vitro tests have shown that polyhydroxybutyrate (PHB) is biocompatible to various cell lines which include osteoblastic, epithelial cell and ovine chondrocytes (Rivard *et al.*, 1996; Nebe *et al.*, 2001). The latest research yielded a good advancement in order to evaluate the biocompatibility of an implanted material by investigating the structural alterations in cellular molecules that are involved in cell adhesion when cells were cultured on PHB (Nebe *et al.*, 2001). Besides that, Rivard *et al.* (1996) reported that when a highly porous well-interconnected PHBV structure was seeded with fibroblasts, it kept the rate cell proliferation similar to that observed in collagen sponges for 35 days, with a maximum cell density being noted on the twenty-eighth day. Nonetheless, the polymers have also resulted in both prolonged acute inflammatory responses and chronic inflammatory responses (Akhtar, 1990).

It is worth noting that, *in vivo*, when most PHA polymers break down, they release hydroxyl acids that are less acidic and less inflammatory compared to many currently used synthetic absorbable polymers (Williams and Martin, 2002).

2.12 Production of PHAs using saponified palm kernel oil as a carbon substrates

Oil palm (*Elaeis guineensis* Jacq) is a perennial monocot belonging to the family Palmae and tribe Cocoineae. It gives the highest oil yield per hectare of all

the economic oil crops (Corley and Tinker, 2003). It is an important crop for Malaysia and has great contributioin to the national economy as Malaysia is one of the main exporters for palm oil (Yusof, 2002). Diversified uses for palm oil are constantly sought such as the conversion of palm oil into high-value and versatile products. Tan *et al.* (1997) had shown that saponified palm kernel oil was able to support cell growth of *Pseudomonas putida* and the production of mcl-PHA. An integral aspect in the production of this material using SPKO as carbon substrates is to study its potential use in the maxillofacial fixation and its potential as a bone substitute.

2.13 Objectives of study

Clinical problems requiring bone regeneration in oral and maxillofacial region are diverse and challenging. These bone-related problems usually require substantial bone grafting surgeries. For example, mandibular fractures and osteotomies have been previously managed with biologically stable metal devices and recently with biodegradable devices. Besides that, guided bone regeneration using the non-degradable membrane is still often applied in the treatment of periodontisis. Stiff non-biodegradable implants may cause osteopenia, cortical porosis, and atrophy of the underlying bone, at least in diaphyseal bone. Therefore, there is a necessity to remove these metal or non-degradable implants in order to prevent the high possibility of infection and weakening of bone which will lead to a possibility of refracture. Biodegradable implants can accommodate the effective processes of bone healing. Over time, the entire material is expected

to disappear completely because of degradation, and thus no secondary surgery is required (Ueda and Tabata, 2003).

As mentioned earlier, PHA and its composites are thought to have good potentials as emerging materials in the medical field for example, to be used as suitable scaffolds in tissue engineering. Most of the research was focused on medical applications of scl-PHA due to its higher crystallization rates and the similar properties to polypropylene (Zinn *et al.*, 2001), as compared to the mcl-PHA, which may be due to the limited availability of mcl-PHA.

In my research, the mcl-PHA which was produced by bacteria using SPKO as carbon substrates was used to investigate its possible osteoconductive properties in bone using rabbit mandible as the experimental model. The emphasis would be to investigate its potential to be used as a substitute for bone graft in oral and maxillofacial region. Therefore, the objectives in this research are as follows:

- To compare the percentage of bone fill in the bony defect with mcl-PHA membrane in comparison to the empty defect.
- To evaluate the quality and quantity of the bone regeneration with the mcl-PHA using histomorphometry.
- To determine the biocompatibility of the mcl-PHA in terms of the response of host tissue.