HYDROTHERMAL CONVERSION OF MALAYSIAN CORAL TO HYDROXYAPATITE, A BONE SUBSTITUTE: SYNTHESIS, CHARACTERIZATION AND *IN-VITRO* SOLUBILITY STUDIES

by

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Thesis submitted in fulfillment of the requirements for the degree of Master of Science

AUGUST 2005

ACKNOWLEDGEMENTS

I am thankful to the Great Almighty, Allah S.W.T. upon the completion of this thesis. First of all, I would like to express my deepest gratitude to my parents, Samsudin Yusoff and Rohani Abdullah who encouraged me to pursue my MSc. Degree.

A special appreciation goes to my supervisor, Dr. Bassim H. Hameed for the wonderful supervision, guidance, encouragement, support and advice throughout my research. Also to my co-supervisors, Prof. Ab. Rani Samsudin and Dr. Mohd Roslee Othman, thank you very much for the unending help and precious opinions. Thousand thanks to Dr. Suzina Sheikh Abd. Hamid, Prof. Razali Othman and Prof. Zainal Ariffin for their comments, guidance and encouragements during the study period. Their brilliant ideas, suggestions and assistance have contributed much in realizing this thesis.

My best regards to Universiti Sains Malaysia (USM) especially to our respected Dean, Deputy Dean, lecturers and all staff of School of Chemical Engineering for their help, support and motivation. My gratitude also goes to the technicians in X-Ray Powder Diffraction (XRD) unit, School of Physics, X-Ray Fluorescence (XRF) unit, School of Material and Mineral Resources, Scanning Electron Microscope (SEM) unit, School of Life Sciences and Fourier Transform Infrared (FTIR) unit, School of Chemistry, USM. Special thanks also goes to all staff at the National Tissue Bank and School of Dental Sciences, USM Medical Campus.

I would also like to address my gratitude to the Ministry of Science, Technology and Innovation for the Long-term IRPA Grant and awarding me with the National Science Fellowship Scholarship (NSF) throughout the entire study period.

Last but not the least, I wish to thank all my beloved friends for their moral support, sharing of knowledge, cooperation and guidance. Finally, for those who have directly and indirectly helped me in this project, thank you very much.

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LIST OF SYMBOLS

		Unit
n	Integer	
d	Distance between crystal plane	Å
Greek letters		
α	Alpha phase	
β	Beta phase	
λ	Lambda	
θ	Theta phase	

LIST OF ABBREVIATION

ASTM	American Society for Testing and Materials
BCP	Biphasic calcium phosphate
BMPs	Bone Morphogenic Proteins
COM	Coral organic matrix
CHA	Coralline-hydroxyapatite
DAP	Diammonium hydrogen phosphate
DCPA	Dicalcium phosphate Anhydrous
EDX	Energy Dispersive x-ray
FTIR	Fourier Transform Infrared
GMA	Glycidylmethacrylate
GAG	Glycosaminoglycan
HA	Hydroxyapatite
IR	Infrared
ICDD	International Centre for Diffraction Data
JCPDS	Joint Committee on Powder Diffraction Standards
MCPM	Monocalcium phosphate monohydrate
PVA	Polyvinyl alcohol
PVB	Polyvinyl butyral
PBS	Phosphate buffer saline
SEM	Scanning Electron Microscope
THR	Total Hip Replacement
TCP	Tricalcium phosphate
TGF	Transforming growth factors
TGA	Thermogravimetric analysis
XRD	X-ray diffraction
XRF	X-ray fluorescent

PENUKARAN HIDROTERMA BATU KARANG MALAYSIA KEPADA HIDROKSIAPATIT, TULANG GANTIAN: SINTESIS, PENCIRIAN DAN KAJIAN KETERLARUTAN *IN-VITRO*

ABSTRAK

Tujuan kajian ini adalah untuk mensintesis hidroksiapatit, $Ca_{10}(PO_4)_6(OH)_2$ daripada batu karang Malaysia spesis *Porites lutea*, untuk digunakan sebagai tulang gantian dalam perubatan dan pergigian. Batu karang telah dicirikan menggunakan pembelauan sinar-x (XRD), jelmaan fourier infra-merah (FT-IR), pendarfluor sinar-x (XRF), analisis permeteran gravity haba (TGA) dan mikroskop imbasan elektron (SEM). Kajian pencirian melibatkan penentuan fasa batu karang, kandungan unsur, morfologi dan kestabilan terma batu karang yang digunakan dalam kajian ini. Pencirian telah dilakukan untuk memastikan batu karang yang digunakan dalam kajian ini adalah bahan mentah yang sesuai. Keputusan pencirian menunjukkan bahawa batu karang terhasil daripada 97.76% kalsium karbonat dalam fasa aragonit, manakala baki 2.24% dimiliki oleh unsur-unsur minor dan surih. Analisis terma batu karang menunjukkan bahawa kira-kira 44% pengurangan berat berlaku pada 791°C yang sama dengan kalsium karbonat komersil. Batu karang spesis ini memiliki struktur berliang yang tersaling hubung diseluruh rangka. Ia mempunyai saiz liang yang seragam iaitu kira-kira 117 μm. Batu karang telah berjaya ditukarkan kepada hidroksiapatit, melalui tindak balas hidroterma. Tindak balas hidroterma telah dijalankan di dalam reaktor kelompok bertekanan tinggi pada suhu di antara 150°C – 250°C dan tekanan 34 atmosfera, yang dijanakan oleh wap air. Banyak parameter yang telah dikaji untuk mencari keadaan optimum pembentukan hidroksiapatit, seperti suhu tindak balas (150°C – 250°C), masa sentuh (1 – 42 jam), jumlah DAP (pengurangan dan lebihan daripada nisbah molar stoikiometri) dan nilai pH (pH 9 – 11) larutan awal. Pembentukan fasa tunggal hidroksiapatit dengan kestabilan terma yang unggul dan kandungan karbonat yang minima telah diperolehi pada suhu 250°C, 6 jam masa sentuh, 30% lebihan DAP dan

pH 9 larutan awal. Pada keadaan ini, pembentukan fasa-fasa lain seperti kalsium hidroksida dan kalsium karbonat tidak dapat dikesan. Kajian kestabilan in-vitro hidroksiapatit telah dilakukan dalam penimbal air masin berfosfat (PBS) dan air ternyah-ion pada 37°C untuk menentukan keterlarutannya dalam keadaan fisiologi. Hidroksiapatit yang dihasilkan pada keadaan optimum adalah yang paling stabil apabila direndam dalam kedua-dua larutan selepas 55 jam.

HYDROTHERMAL CONVERSION OF MALAYSIAN CORAL TO HYDROXYAPATITE, A BONE SUBSTITUTE: SYNTHESIS, CHARACTERIZATION AND *IN-VITRO* SOLUBILITY STUDIES

ABSTRACT

The purpose of this study is to synthesize hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ from Malaysian coral of species *Porites lutea,* to be used as bone substitute in medicine and dentistry. Coral was characterized using x-ray diffraction (XRD), fourier transform infra-red (FT-IR), x-ray fluorescence (XRF), thermogravimetric analysis (TGA) and scanning electron microscope (SEM). The characterization study involves the determination of coral phases, element content, morphology and thermal stability of coral used in this study. The characterization was carried out in order to ensure that the coral used in this study was suitable raw material. Characterization results revealed that coral was made up of 97.76% calcium carbonate in aragonite phase, while the remaining 2.24% belongs to minor and traces element. Thermal analysis of coral showed that about 44% weight loss occurred at 791°C which was identical to that of commercial calcium carbonate. Coral of this species possessed porous structure with interconnectivity throughout the skeletal. It has a consistent pore size of around 117μm. Coral was successfully converted into hydroxyapatite, through hydrothermal reaction. Hydrothermal reaction was carried out in a high-pressure batch reactor at temperature range between 150°C – 250°C and pressure of 34 atmospheres, generated by water vapor. Many parameters have been studied in order to find the optimum conditions of hydroxyapatite formation, such as reaction temperature (150°C – 250°C), contact time (1 – 42 hours), amount of DAP (less and excess than stoichiometric molar ratio) and pH value (pH 9 -11) of initial solution. The formation of single phase hydroxyapatite with excellent thermal stability and minimal carbonate content was obtained at 250°C, 6 hours of contact time, 30% excess of DAP and at pH 9 of the initial solution. At this operating condition, the formation of other phases such as calcium hydroxide and

calcium carbonate were not detected. The in-vitro stability studies of hydroxyapatite were performed in phosphate buffer saline (PBS) and de-ionized water at 37°C to determine its solubility in physiological environment. Hydroxyapatite synthesized at optimum conditions was the most stable when immersed in both solutions after 55 hours.

CHAPTER ONE
INTRODUCTION

1.1 Biomaterial

Use of biomaterials is one of the emerging field that is growing rapidly to meet the demand in medicine and dentistry. Over the past few decades, new biomaterials such as bone replacement, total hip prosthesis and dental implant have been synthesized, designed, fabricated and commercialized for various needs. Currently, thousands of these materials can be found easily in the market. The market for orthopedic biomaterials, including bone replacement was worth over $1 billion in 2004 for United States only. The European orthopedic biomaterial market, comprising France, Germany, Italy, Spain and United Kingdom was valued at almost $200 million in 2004 (Millennium research group, 2005). The market for orthopedic biomaterial is expected to increase each year due to the need for better solution to damage and fracture due to injuries, disease and aging population all over the world.

In Malaysia, orthopedic biomaterial research especially in bone replacement is still in the earliest stage. Bone loss and fracture due to accident either on the road or in the workplace needs to be replaced with new ones in order to ensure functionality, and to impart quality to life. As a result, the demand for bone replacement increases with the society's awareness of healthy life style. Apart from that, the need for bone substitute has also increased to treat patients who suffer from bone disease, such as osteoporosis. As bones age, they loose the mineral content and this will lead to bone fracture. Recent development in reconstructive surgery especially for bone replacement has somehow created another problem that has not been solved satisfactorily. Large bone defect caused by major injuries, trauma or imperfections needs to be treated with a large amount of bone substitute.

Although the requirement for bone replacement is increasing in Malaysia, the materials need to be imported from other countries such as United State, German and Switzerland. Imported materials are more expensive and sometimes cannot be afforded by local community. For example, the price for 1 cm^3 bone graft was reported to reach RM1,500 (Chandrasekaran & Mustafa, 2003). Generally, in surgery the amount of graft needed is high. Malaysia has spent more than RM30 million each year to buy synthetic bone graft from foreign companies. It was also noted that about 30,000 orthopedic surgeries with estimated cost of RM150 million conducted every year in Malaysia (Chandrasekaran & Mustafa, 2003).

Traditional bone substitutes (from natural origin) which include autograft, allograft and xenograft are commonly used to treat bone defects. Among these, autograft is accepted as a gold standard for bone graft due to its highly osteogenic capacity (Gogolewski, 2001). However, the use of bone substitute taken from natural origin, either human or animal origin is not a favorable one since it resulted in various other problems, such as infections, immunogenic response, disease transmission and limited supply when involving a large scale surgery (Vaccaro *et al.*, 2002; Sandor, 2003). Furthermore, the processing and storage of these bone graft are quite expensive.

Due to the high demands for bone substitutes, many researches have been conducted to produce synthetic bone substitute in large quantities (Saeri *et al.*, 2003; Jinawath *et al.*, 2002). Synthetic bone graft such as ceramic, polymer and metal were introduced as an alternative to the traditional bone substitute. Among these materials, calcium phosphate ceramic, especially hydroxyapatite is the most suitable material due to excellent biological properties and its chemical compositions is similar to human bone. Hydroxyapatite, in the form of powder, dense or porous material is used to repair bone and periodontal defects, eye implant and as a coating material for metallic

implants. Although the use of synthetic hydroxyapatite could solve the problems faced by traditional bone graft, it has never matched the quality of natural material. Many researches have been conducted to synthesize hydroxyapatite through different methods (Zang *et al.*, 2001; Anee *et al.*, 2003; Liu *et al.*, 2003). Most of these methods have been produced through chemical reactions. However, there is still a lack of research done to synthesize hydroxyapatite from natural sources. The production of synthetic hydroxyapatite using natural material, such as coral is hoped to gives better alternatives to the healing process in bone surgery.

Coral is a marine organism that prefers to lives in warm and shallow water. Coral of genus *Porites* can be found abundant in Malaysia coast and was harvested for the purpose of this study. It has a porous structure with interconnectivity throughout the skeletal which is mimicking the structure of trabecular bone.

1.2 Problem statement

Malaysia has a wide variety of corals of different species and shapes, but very limited research has been done on their potential as a bone substitute. Coral of genus *Porites* seems to be a promising material due to its biocompatibility, biodegradable and porous structure with interconnectivity throughout the skeletal (Fricain *et al.*, 2002; Braye *et al.*, 1996). As a natural material, coral is readily accepted by the body without causing any negative response. It contains similar mineral found in human bone, such as calcium, magnesium and other essential minerals (Pszczola, 2003). Recently, certain species of coral has been commercialized as a calcium supplement to treat diseases related to calcium deficiency (Pszczola, 2003). In medicine and dentistry, sea corals are widely used as an implant for the reconstruction of bone defect in humans and animals. It is mostly used to correct periodontal defect in maxillofacial surgery, as filler for bone fracture and also to reconstruct bone defects in cranial surgery. Many studies were carried out in order to find the materials most suitable that can be used as

bone substitute. Corals have currently become an ideal material because it is affordable, readily available in large quantities.

Coral can be found abundant in our diversity and harvesting them for the purpose of this study does not give any negative impact to the environment. Since coral is a protected marine life, harvesting was only done three times a year with a limitation of 5 kilograms of corals taken during each visit. A permit was issued by the Fisheries Department that gives permissions to USM biomaterial research group to collect only the dead corals which already detached from the reefs. Furthermore, this activity does not disturbed marine ecosystem and the amount of coral needed to make bone substitute has a negligible effect on the environment.

Although coral can be used as a bone substitute it still has weaknesses that limit its application. Coral contains similar mineral found in bone, but the composition of these mineral is slightly different from bone. Human bone consists mainly of hydroxyapatite, while coral is made up of calcium carbonate. The resorption rate is much faster in coral than hydroxyapatite, which make it lost before the bone tissue can completely grow (Braye *et al.*, 1996). In addition, porous hydroxyapatite has better ability to induce bone formation and accelerate the healing process as compared to coral. To the best of our knowledge there are no previous reports on hydroxyapatite synthesized from coral species found in Malaysian coast. Thus, the objective of this work is to synthesize hydroxyapatite as the most suitable bone replacement from natural coral through hydrothermal technique. Many experimental factors, such as reaction temperature, contact time, phosphate ratio and initial pH value have been considered to ensure the hydroxyapatite obtained is of high quality and excellent properties. Besides chemical and physical properties, in-vitro stability is also one of the crucial factors investigated in order to determine whether the obtained hydroxyapatite

could be used as a bone substitute. The production of hydroxyapatite under hydrothermal condition could provide a sterile environment during the reaction.

1.3 Research objectives

The present research has the following objectives:

1. To characterize the coral of *Porites lutea* collected from the Malaysian coast.
2. To synthesize hydroxyapatite from the coral and find the optimum operating conditions. The hydrothermal process was conducted with various parameters such as reaction temperature, contact time, amount of diammonium hydrogen phosphate and pH of initial solution.
3. To characterize the synthesized hydroxyapatite obtained from hydrothermal process.
4. To study the in-vitro solubility of synthesized hydroxyapatite and compare it with commercial hydroxyapatite.
5. To fabricate a high-pressure batch reactor (medical grade) for hydrothermal conversion of Malaysian coral into hydroxyapatite, a bone substitute.

CHAPTER TWO
LITERATURE REVIEW

2.1 Structure and properties of bone

Human bones and teeth are the most studied tissue compared to other tissues. It is a large tissue in the body and carried many specific functions such as (Graaff and Rhees, 2001):

i. as a support for soft tissue attachment
ii. maintain the body strength in load applications
iii. helps to protect the vital structure of soft tissue
iv. productions of white and red blood cells and platelets
v. storage of minerals especially calcium and phosphorus
vi. gives the mechanical basis for body movement

Figure 2.1 shows the structure of long bones (Spence, 1990). The long bone consists of two parts, that is, the diaphysis, which is the body or shaft of a long bone, and the epiphysis, which is its enlarged head. The outer shell and the shaft of a long bone consist of compact bone, while at the epiphysis, it consist of spongy bone. The bone is covered by a thin layer of vascular fibroelastic connective tissue, known as periosteum. The periosteum is made up of two layers. The outer layer is called a fibrous layer. It contains a large number of collagenous fibers, some blood vessels, and nerves. The inner layer is called cellular layer, consist of many connective tissue cells, including precursor cells for osteoblasts (Han and Holmstedt, 1981).

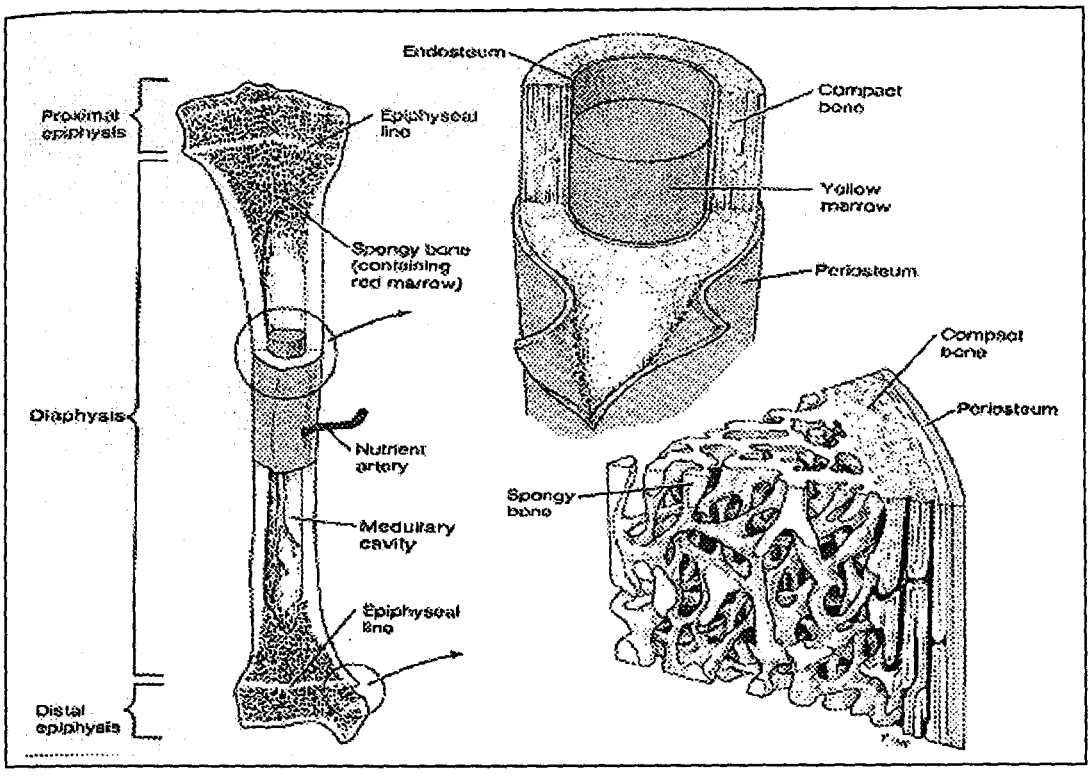


Figure 2.1 Structure of long bones (Spence, 1990)

Human bones can also be divided into two different phases, organic and inorganic. The main inorganic part consists of nano-crystalline hydroxyapatite in the form of plate or needles, about 40 nm long, 20 nm wide and 5 nm thick (Gunderson and Schiavone, 1991). Other minerals including calcium carbonate and trace amount of sodium, magnesium, potassium, fluorine, chlorine, pyrophosphate and hydroxyl ions are also present (LeGeros, 1994). These minerals give the strength and hardness to the bone. The inorganic part forms compact and spongy bones. These two types of bones are classified based on their degree of porosity and microstructure. Each of these bones is built from many cylindrical channels called osteons, which is held together by natural hydroxyapatite (Engin and Tas, 2000). At the centre of these osteons is a hollow canal that contains the blood vessel and nerves systems.

The major organic part of bone is collagen in forms of nano fibres. It provides a place for the mineral especially hydroxyapatite to deposit within these fiber. Bone also

contains cellular components, such as osteoblast, osteoclast and osteocytes (Han and Holmstedt, 1981). Each of them has specific functions towards bone formations and regenerations. Other organic materials are also present in bone in small quantities, such as proteins, polysaccharides and lipids (Gunderson and Schiavone, 1991). Bone also consists of red and yellow marrow. Red bone marrow is highly vascularized and active in blood formation. It will be replaced by yellow marrow as the bone ages (Han and Holmstedt, 1981). The organic part plays an important role to initiate immune response towards foreign materials.

The strength of hard tissue is mostly influenced by the degree of mineralization or the density of the bone. Mineral content gives the hardness and prevent bone fracture. Increased level of bone mineral will increase the strength and stiffness of the bone (Follet *et al.*, 2004). Organic component, mainly collagen would behave as a compliant material with high toughness, low modulus and other properties characteristic for polymers. High toughness is also related to the presence of other complex fibrous microstructure.

2.2 Bone graft

Grafting is one method for surgical transplantation or implantation in bone replacement. Bone graft is commonly used to:

i. Replace damaged bone

   Diseased bone has to be removed and the gap needs to be filled with a certain material either natural bone graft or custom-made prosthesis.

ii. Stimulate growth of the bone cells from the host and speed up the healing process.

iii. Provides support for the cells to attach and grow into three dimensional structures.

Bone graft made from biological or natural origin is different from the engineered or synthetic graft. Biological materials, such as, tissue contains cells and have the ability to repair themselves, whereas, synthetic materials do not. Natural bone substitute can be divided into three groups, known as autograft, allograft and xenograft. The use of natural bone replacement may cause many problems, such as infection, disease transmissions, morbidity and pain at the donor site. These problems have limited the use of natural bone graft. Therefore, synthetic bone substitute was developed as an alternative to the traditional bone replacement.

Autogenous bone grafting is a standard procedure for bone transplant due to its highly osteogenic capacity. This procedure ensures the absence of immunogenic responses and the bone graft's biocompatibility with natural tissue. (Gogolewski, 2001). Bone is harvested from the donor site and transplanted into the defect area of the same patient. Fresh autograft harvest from patient may contain viable stem cells and osteoblasts that contribute to the bone fusion process. It also contains several osteoinductive factors, such as bone morphogenic proteins (BMPs) that are very important to enhance bone growth (Vaccaro *et al.*, 2002). The major disadvantage of autograft is that it requires additional surgery and high operating cost. Harvesting the graft also causes morbidity to the donor site, such as pain and infections (Gogolewski, 2001). The use of autograft is also limited to minor bone defect because of limited supply. When involving major reconstruction of bone defect, the graft is not enough and therefore, need to be used together with other material which would act as an extender.

Allograft is introduced as a solution for the autogeneous procedure since it ensures the right amount of graft needed. In this procedure, tissue is transferred between two genetically different individuals. Bone tissue harvested from donors is processed and stored properly in the tissue bank before being used in surgery in order to reduce contaminations. However, fresh grafts and fresh frozen grafts are rarely used

because of the risk of disease transmission. Freeze-dried bone has lower immunogenicity and low osteoinductive properties thus resulting in slower remodeling (Vaccaro *et al.*, 2002).

Another type of bone grafting is xenograft which involves transplants of bone between two different species. This graft is usually harvested from mammals, such as bovine and porcine. Xenograft is preferable because it is possible to harvest larger amounts of bone as compared to bone taken from human origin. Similar to allograft, xenograft also undergoes extreme chemical processing and irradiations to reduce the immunogenecity and contamination, thus resulting in a lower osteogeneic capacity. However, the use of this graft has the same problem which is the development of immunogenic response (Begley *et al.*, 1996).

Xenograft can also be derived from exoskeleton of scleractinian corals. Commercially available coral implant (Biocoral®, Inoteb, LeGuernol, Saint-Gonnery, France) has been obtained from calcium carbonate exoskeleton of scleractinian coral. Calcium carbonate in the form of aragonite accounts for more than 97% of the weight. Other trace element also exists in an equal amount to the mammalian bone. Biocoral has porosity of around 50% volumes, with pore sizes ranging from 150 to 500 microns and an interconnecting fenestration throughout the entire substratum (Gao *et al.*, 1997). Gao *et al.* (1997) implanted cylindrical samples of biocoral inside twelve adult female sheep. They found that new formed bone have penetrated into the biocoral substratum after implantation. However, the degradation of biocoral was not consistent in all models (Gao *et al.*, 1997). The use of coral as a bone substitute could reduce the morbidity of donor site and the graft is available in large quantities.

Synthetic bone grafts which include metals, ceramics and polymers are widely used in medicine and dentistry. However, not all the materials in these classes can be

use as an implant. There are certain requirements that have to be met for any replacement materials namely: biocompatibility, appropriate mechanical properties, no inflammatory response, not carcinogenic and not toxic. They should also be able to promote bone growth, undergoes biodegradation after fulfilling their functions and easily formed into the desired shape (Gogolewski, 2001; Shors, 1999).

Metal is used in many bone surgery where mechanical support is needed. However, long term exposure to the biological environment and frictions between metal surface and the tissue leads to failure. Failed implant needs to be replaced with new one through surgery and this involves high operation cost. Ceramic is use as alternatives for metal implant since it provides better biological interactions with living tissue. The most commonly used ceramics is hydroxyapatite because of its bioactivity and similar compositions to bone. Different ceramic have different mechanical and biological properties. Another method is by using biodegradable polymers, such as polyethylene to fill bone defects and as a carrier for growth factors. Similar to ceramics, polymer graft will resorb, and at the same time new bone grows inside the voids (Ovaginian, 2001).

Total hip replacement (THR) is a serious health requirement almost in every country around the world. Over 150,000 total hip replacement procedures are undertaken annually in United State (Katti, 2004). However, this implant fails after a certain period and must be replaced, normally after 15 years. It is estimated that 20% of hip replacement surgery simply replace the original, failed implant (Katti, 2004). Total hip replacement is made from a combination of metal, ceramic and polymer. This combination gives excellent strength and bioactivity to the implant.

Although these biomaterials are widely used, it can never match the quality of autograft. Synthetic materials do not contain viable cells, thus the regeneration of new

tissue is difficult to obtain. New approach towards regenerations of tissue is by seeding the synthetic implant with a living cell or growth factors. This will allow the cells to multiply and grow into three-dimensional tissues, and recreate their specific tissue functions. After a certain time, blood vessel will attach to this implant and new tissue will form. Due to the high demands for bone substitute, continuous research is being conducted to increase the quality of biomaterials. The productions of synthetic bone graft using local materials are hoped to give better alternatives to the healing process and thus, improving the quality of life.

2.3 Natural sea coral

Coral is a marine life form that have a wide spread of habitat in the oceans around the world. Corals are classified under the Phylum *Cnidaria,* in the class of *Anthozoa*, and in the Order *Scleractinia* (Roberts, 2004). Corals grow in large bunches with many different species, colors, forms and sizes. It can be found mostly in the tropic areas between the latitudes of 25° north and 25° south of the equator (Henry, 1982). In this area, the sun shines directly, heating the land and surface layer of the oceans. Corals prefer to live in warm water and they can build up the reefs only between water temperatures of about 18 to 30°C (Sheppard, 1983). At this temperature, they can build the skeleton at a sufficiently fast rate. Besides the right temperature, most of the reefs-building corals are found in shallow water. The suitable depth is reported to be less than 35 meters deep (Henry, 1982).

Coral reefs or skeleton are a complex structure that is built from billions of tiny animals called coral polyps. Through a certain process, it can build a very large and complex structure. The main material that constructs the inorganic skeleton is calcium carbonate which is plentiful in the oceans. Polyps extract calcium carbonate from their surrounding and deposit it in their skeletons through a process similar to the calcification process used by mammals to deposit enamel on the dentine of teeth

(Shors, 1999). When the polyps die, their skeleton will be used by new polyps as a base to grow and they also secrete calcium carbonate and build the coral stone above the layer of the old one. The colonies of polyps only occupy the top layer of coral skeleton (Sheppard, 1983).

Corals provide shelter and food for other marine life in the ocean. They help remove excess gases, such as carbon dioxide, by taking calcium carbonate from the sea (Roberts, 2004). Coral is harvested from our diversity for many purposes. In medicine and dentistry, corals are widely used as an implant for the reconstruction of bone defect in human and animal. The need for bone replacement arises from many reasons such as diseases, aging, infections, accidents, trauma or imperfections.

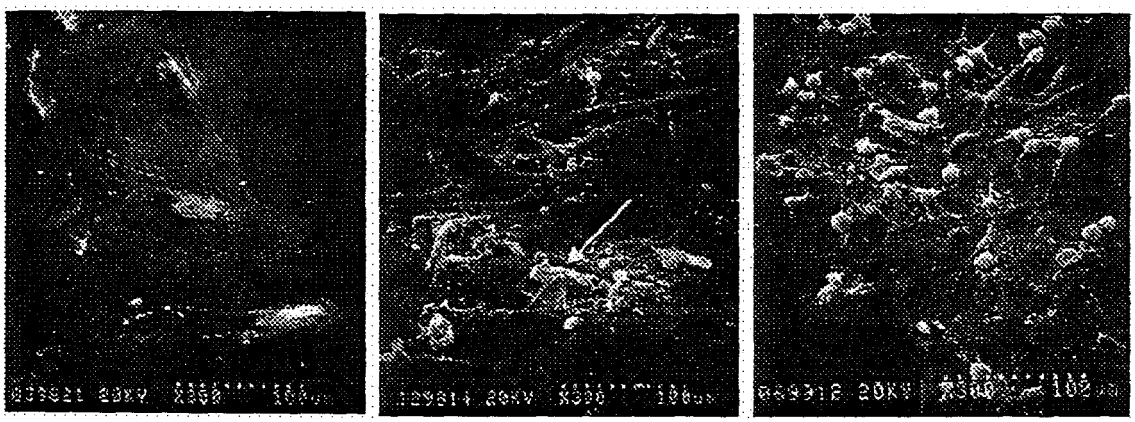
A few species have been identified and studied as possible bone substitute. The following genera have already been used as bone graft, that is, *Pocillopora, Acropora, Montipora, Porites, Goniopora, Fungia, Polyphyllia, Favites, Acanthastrea, Lobophyllia* and *Turbinaria* (Bouchon *et al.*, 1995). As a natural material, coral is readily accepted by the body and available in a large quantity.

2.4 Bone replacement from sea coral

The most commonly used coral as a bone implant is in the Genus *Porites* and *Goniopora* due to their exoskeleton structure which is mimicking the structure of human cancellous bone (Vuola *et al.*, 1998; Hu *et al.*, 2001). Other genus might not be very popular mostly because of its structure and mechanical strength. The dead coral is usually cleaned and undergoes chemical treatment and irradiation to eliminate the organic matrix and impurities before it can be used as an implant.

Coral skeletal consists of 99% calcium carbonate (mostly in the form of aragonite and sometime calcite) and 1% organic material (Vuola *et al.*, 1996). The

remaining organic materials which include the Glycosaminoglycan (GAG), protein and carbohydrate are present in the organic matrix (COM) of *Porites astreoides*. The cytocompatibility study on human bone marrow cell culture showed that the COM has a negligible cytotoxic effect (Fricain *et al.*, 2002). Certain species of *Scleractinian* coral also consisted of lipid and chitin in their organic matrix (Watanabe *et al.*, 2003). However, this substance was not further studied for coral that use as bone substitute. Figure 2.2(a) shows the SEM photograph of human bone marrow cell culture placed as a control, while Figure 2.2(b) and 2.2(c) shows the SEM photography of human bone marrow cell culture in contact with COM particles (Fricain *et al.*, 2002).



(a) (b) (c)

Figure 2.2 SEM photography of human bone marrow cell culture in contact with COM particles (Fricain *et al.*, 2002)

Figure 2.2 (b) shows the human bone marrow cells after 24 hours of culture in contact with COM particles. These cells have morphology similar to those observed with the control after 24 hours as shown in Figure 2.2 (a). Figure 2.2 (c) shows the cell in the present of 160μg COM after 72 hours. At this concentration of COM, the cells seem to be dead (Fricain *et al.*, 2002).

Coral pre-treatment process has changed the properties of the organic matrix and makes it safe for clinical use. Coral without its organic matrix could reduce the

possibility of inflammatory response. The in-vivo study showed no rejection or fracture of the coral after being implanted in the cortical bone of the femur's diaphysis (Braye *et al.*, 1996).

The microstructure of the natural sea coral is similar to the structure of human cancellous bone. Coral have a consistent pore size which is very important for the growth of fibrovascular and bone tissue. Porous structure provides large surface area compared to dense structure for the cells to attach and form strong bonding with the implant surface (Werner *et al.*, 2002). Besides having macro-pores (150 - 500 μm), coral also consist of nano-pores (5 – 50 nm) within its fibrous structure that makes the solid matter between the pores (Ben-Nissan, 2003). Figure 2.3 shows the surface morphology of *Porites* skeletal (Ben-Nissan, 2003).

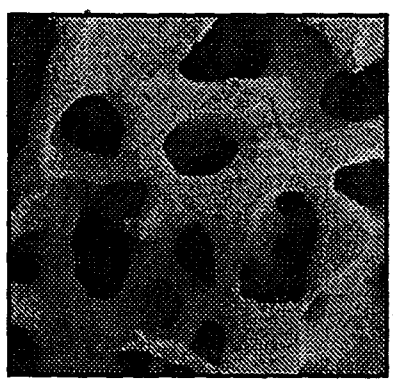


Figure 2.3 SEM of *Porites* skeletal (Ben-Nissan, 2003)

These properties although have contributed to a higher bioactivity but it reduced the mechanical strength. The interconnected porosity of this material allows the blood to diffuse more deeply than in a material of disconnected porosity (Braye *et al.*, 1996). It also provides a place for strong tissue attachment and prevents loosening of the implant during healing process (Jinawath *et al.*, 2002).

Gao *et al.* (1997) noted that new bones formed and penetrated into the coral implant after 3 weeks post-implantation with a larger area and higher density as compared to tricalcium phosphate (TCP). TCP are recently being used as an implant because of its ability to support bone growth and absences of inflammatory reactions. It is suggested that the calcium carbonate in coral have the ability to promote rapid new bone formation from the adjacent host tissue which is not present in TCP. Better osteointegration and mechanical performance were observed in coral implant as against the tricalcium phosphate (Gao *et al.*, 1997).

Degradations of TCP occur rapidly compared to coral which make it loose its strength at a short period after the implantation. However, the resorption rate is much faster in coral (5 months) than for synthetic hydroxyapatite (9 months) under the same experimental conditions (Braye *et al.*, 1996). TCP and coral implant is lost before the bone tissue can completely grow and make it unsuitable for the reconstruction of large defect. An ideal bone substitute should not undergo biodegradations in the body before the tissue completely growth to ensure proper attachment of the tissue to the implant.

It is well documented that coral and other potential bioceramics have excellent osteoconduction properties but lack of osteoinductive capability that unable them to form bone. They only provide good scaffold for the ingrowth of bone from the surrounding tissue but have no ability to form bones themselves. The in-vivo study on rat abdominal musculature shows that no bone formation occurs within the coral implant (Begley *et al.*, 1996). However, bone formation inside the implant can be induced by seeding the implant with bone marrow or by adding growth factors, such as transforming growth factors (TGF-β) to regulate and enhance new bone formations (Vuola *et al.*, 1996; Vuola *et al.*, 1998).

Vuola *et al.* (1996) implanted coral and hydroxyapatite seeded with bone marrow into latissimus dorsi muscle in rat. They reported that new bone formation was found only in implants containing bone marrow. Implant without bone marrow was filled with fibrovascular tissue within the pores. Bone formation was higher in coral than in hydroxyapatite (HA) at 3 weeks (10.8% vs 4.8%) and at 12 weeks (13.7% vs 6.3%, bone/total original block area). However, at 12 weeks the coral implant has resorped and reduced to 40% of the original cross-sectional size. Inflammation reaction was detected in both implants due to the body response towards the foreign material (Vuola *et al.*, 1996).

Further study showed that the compressive strength of coral after implantation was higher than HA since more new bone was formed during the healing process. Although the strength was decreased after the implantation compared to dry coral (coral before implantation), the * value was increased with the longer period of implantation (Vuola *et al.*, 1998). Unimplanted coral have a good mechanical properties as compared to other bioceramic (TCP and HA), but their strength are only comparable with the human cancellous bone (Gao *et al.*, 1997). Bone ingrowth into coral has enhances its compressive strength and it is comparable to that of cancellous bone. However, it is still not sufficient to be used in weight bearing areas.

Although coral exhibit good biocompatibility with the host tissue, it still has several weaknesses that limit the application especially when involved the mechanical loading. Although the microstructure of coral resembles the structure of human bone, but the composition in coral is different. Human bone consists mainly of calcium phosphate, especially hydroxyapatite, while coral consist mainly of calcium carbonate. The resorption rate is much faster in coral than for synthetic hydroxyapatite, which makes it lost before the bone tissue can completely grow (Braye *et al.*, 1996). So, it is unsuitable for the reconstruction of large defect.

2.5 Hydroxyapatite

Hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, is a bio-ceramic material that belongs to the apatite family (also known as calcium phosphate) with substitutions of hydroxyl ion. Other members in this family are fluorapatite, $Ca_5(PO_4)_3F$ and chloroapatite, $Ca_5(PO_4)_3Cl$ (Klein and Hurlbut, 1993). Calcium phosphate biomaterials are polycrystalline ceramics derived from individual crystals of a highly oxidized substance that have been fused together at the crystal grain boundaries by sintering process (Jarcho, 1980). Among calcium phosphates, hydroxyapatite is known to be the major constituents (69%) of bones (Anee *et al.*, 2003). Other forms of calcium phosphate apatite exist as carbonate-fluorapatite and carbonate hydroxylapatite (Smith, 1994). Hydroxyapatite can be synthesized through chemical reaction and can also be found abundant in nature and in living systems.

The crystal structure of hydroxyapatite is shown in Figure 2.4. Basic structure of apatite, $Ca_{10}(PO_4)_6(OH, Cl, F)$, can be described as the three dimensional network of PO_4 tetrahedral which are linked together by the columns of ninefold coordinated Ca1 atoms. The channel passing through the network have the axes coinciding with the sixfold screw axes and contain the triangles of sevenfold coordinated Ca2 atoms and OH^-, F^- or Cl^- ions (Ivanova *et al.*, 2001).

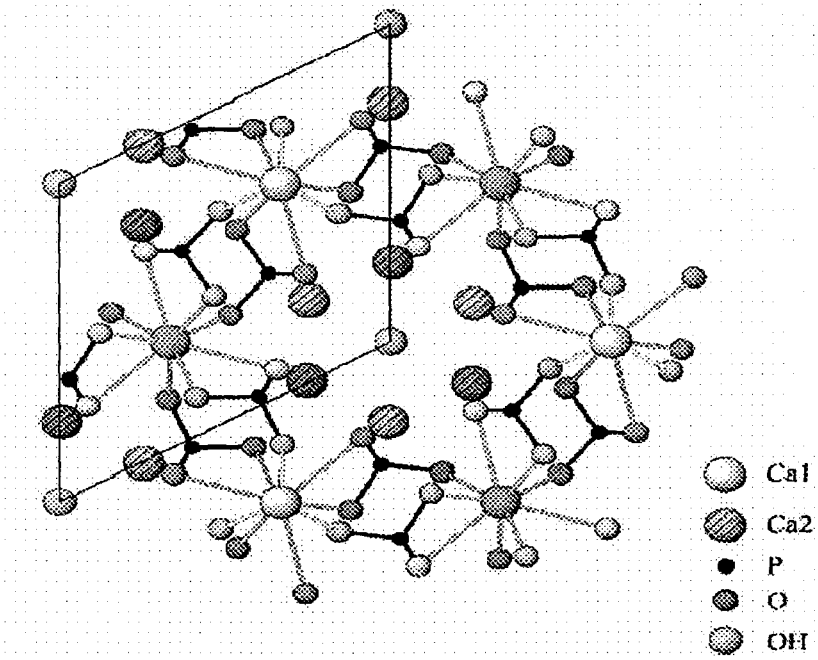


Figure 2.4 Crystal structure of hydroxyapatite (Ivanova *et al.*, 2001)

Biological apatite is different from pure synthetic hydroxyapatite. It is different in composition, crystal size and stoichiometric. Biological apatite can be found as a main inorganic phase in vertebrate bone and tooth and some species of sea shells. In general, biological apatites are non-stoichiometric or calcium-deficient (LeGeros, 1994). It might contain other ions, such as CO_3^{2-}, Mg^{2+}, Na^+, Fe^{2+}, HPO_4^{2-}, F^- and Cl^- in traces (Tadic *et al.*, 2002). All mineral structure in apatite family are based on the fluorapatite with the formula,

$$X_3Y_2(TO_4)Z \qquad (2.1)$$

Where: X and Y are large sites with 7 and 9 coordination, respectively,

T is a 4-coordinated tetrahedral site, and

Z is an anion site in a channel defined by the X sites where its coordination is 3 X cations.

A few substitutions in biological apatite are shown in Table 2.1 (Smith, 1994).

Table 2.1 Crystal chemical substitutions in natural apatite (Smith, 1994)

X, Y	=	Ca, Sr, Ba, Re, Pb, U, Mn and rarely Na, K, Y and Cu
T	=	P, As, V, Si, S and C (as CO_3)
Z	=	F, Cl, OH and O

Calcium phosphate especially hydroxyapatite and tri-calcium phosphate (Ca/P molar ratio of 1.50) are recently investigated as a bone substitute in medical and dentistry because of their excellent biological properties and similar composition to human bone. It is used mostly to repair bone defects, periodontal defects, maintenance or augmentation of alveolar ridge, ear implant, eye implant, spine fusion and adjuvant to uncoated implants (LeGeros, 2005). Synthetic hydroxyapatite for medical purpose must be in ceramic state since it exhibits the biological and medical properties compared to the non-ceramic hydroxyapatite (ASTM, 1999). It must meet the standard requirement in order to ensure the material used is safe.

American Society for Testing and Materials (ASTM) has fixed the standard for compositions of hydroxyapatite used in surgical implant. The concentration of trace elements in the hydroxyapatite is based on standard ASTM F 1185-88 (ASTM, 1999). The standard is shown in Table 2.2.

Table 2.2 Standard specification of trace elements in the hydroxyapatite used for surgical implants (ASTM, 1999)

Element	Maximum concentrations (ppm)
Arsenic (As)	3
Cadmium (Cd)	5
Mercury (Hg)	5
Lead (Pb)	30
Total heavy metals	50

Table 2.3 represents the percentage of impurities existing in the commercial product provided by the supplier (Knepper *et al.*, 1997).

Table 2.3 Analysis of the hydroxyapatite powder (Merck Darmstadt, Germany) (Knepper *et al.*, 1997)

Components of hydroxyapatite powder	Percentage (%)
Analysis (complexometric)	96.2
Impurities Non-soluble in HCl Chloride (Cl) Fluoride (F) Sulphate (S) Heavy metals (As, Pb) Arsenic (As) Iron (Fe)	 <0.05 <0.05 <0.005 <0.25 <0.003 <0.0002 <0.04
Particle size <20μm 2-20μm >20μm	 ≈20 ≈75 <5
Water content	2

Commercial HA can be found in various sizes and forms, either dense, porous block or granules, depending on their applications to repair different bone defects. Dense HA is used to fill defect where mechanical load is required. However, this material has poor porosity which limits the growth and integrations of bone tissue into the implant.

Dense HA was fabricated from nanocrystalline hydroxyapatite powder through compaction techniques followed by sintering process at high temperature between 1000°C to 1200°C (Suchanek and Yoshimura, 1998). Sintering process reduces the pores between particles and produces high density HA having high mechanical strength. Dense HA with excellent mechanical properties can be achieved when the HA powder is stoichiometric, that is having a Ca/P molar ratio of 1.67 (Liu *et al.*, 1997). If the HA powder is non-stoichiometric, the formations of other calcium phosphate such as tricalcium phosphate (TCP), $Ca_3(PO_4)_2$ may occur during sintering which will reduce the strength and other properties (Ruys *et al.*, 1995). In order to obtain ceramic containing pure HA, the decomposition must be avoided.

HA implant with high porosity has gained more acceptances due to its ability to promote bone growth into the porous structure. Porous hydroxyapatite has better integration with host tissue that helps to hold the material in the defect and avoid loosening of the implant. This bond formation has also increased the strength of the implant over time (Vuola *et al.*, 1998). Classical method to prepare porous HA is by mixing the HA powder with combustible material and sintered at elevated temperature to create pore. Liu (1996) previously synthesized HA granules with controlled porosity and consistent pore sizes by adding poly(vinyl butyral) powders (PVB) into a slurry of pure HA and heated at 500°C to generate the pores.

Porous hydroxyapatite can also be synthesized from mammals bone by sintering process. Figure 2.5 shows the SEM of hydroxyapatite granules derived from bovine bone (Joschek *et al.*, 2000).

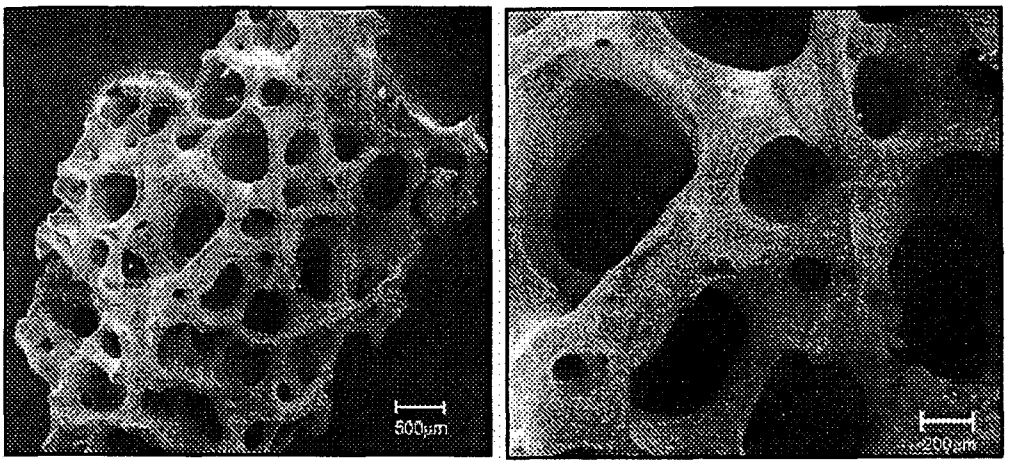


Figure 2.5 SEM of hydroxyapatite granules derived from bovine bone (Joschek *et al.*, 2000)

Hydroxyapatite derived from animal bone exhibits the porous structure identical to natural bone itself. The structure of bovine bone was preserved after the conversion into hydroxyapatite, as shown in Figure 2.5. The obtained HA was very porous with porosities of 57 ± 2%. High porosity is very important to ensure a large amount of drugs can be incorporated into the granules when used as a drug carrier (Joschek *et al.*, 2000). There are two types of pores within these granules which are macropores with an average size of approximately 300 µm and micropores with average size of approximately 1.3 µm (Joschek *et al.*, 2000). The micropores in these granules seem to be less important than macropores if the granules are to be used as a drug carrier (Joschek *et al.*, 2000). However, it can contribute to the bioactivity for bone cells attachment.

2.6 Preparation of hydroxyapatite

2.6.1 Wet precipitation process

Precipitation method is the most commonly used to synthesize high purity and stoichiometric hydroxyapatite (HA) powder with the reaction temperature lower than 100°C. This process includes the calcination procedures (at temperature of 800 to 1000°C) of precipitated powder to obtain a stoichiometric structure. There are different

chemical reagents that can be use in this technique, but the two most popular is through the reactions of (1) calcium nitrate and diammonium hydrogen phosphate (Engin and Tas, 2000; Zang *et al.*, 2001) and (2) calcium hydroxide with phosphoric acid (Kweh *et al.*, 1999; Lazić *et al.*, 2001; Saeri *et al.*, 2003). The first method of reaction was based on the following equation:

$$10Ca(NO_3)_2 + 6(NH_4)_2HPO_4 + 8NH_4OH \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 20NH_4NO_3 + 6H_2O \qquad (2.2)$$

The disadvantage of using this method is that the purity of the precipitated HA depended on the purity of calcium nitrate used. The second method is preferred than the first method because in this reaction the only by-product is water (Kweh *et al.*, 1999). Kweh *et al.* (1999) synthesized HA powder using orthophosphoric acid and calcium hydroxide based on the following equation:

$$10Ca(OH)_2 + 6H_3PO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 18H_2O \qquad (2.3)$$

The precipitate was transported to the spray drier to be atomized into fine particles. Spray drier is used to produce homogenized spherical powder of HA with specified sized distributions. The powder obtained exhibited only the HA phase with the absence of other calcium phosphate or undesired phases. The spray-dried HA powder was calcined at 900°C for 2 hours to remove the PVA binder and densify the powder. Hydroxyapatite obtained before calcinations exhibited low degree of crystallinity. Kweh *et al.* (1999) noted that the desired temperature for calcination of HA was below 1000°C, since the presence of TCP was detected at this temperature and higher. Formation of other calcium phosphate is undesirable because it could result in the unreliable stability when used as a coating (Kweh *et al.*, 1999).