SCHOOL OF MATERIALS AND MINERAL RESOURCES ENGINEERING UNIVERSITI SAINS MALAYSIA

FISH BONE WASTE AS POTENTIAL SOURCE OF APATITE FOR BONE REPLACEMENT

By

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Dissertation submitted in partial fulfillment
of the requirements for the degree of Bachelor of Engineering with Honours
(Materials Engineering)

Universiti Sains Malaysia

JUNE 2018

DECLARATION

I hereby declare that I have conducted, completed the research work and written the dissertation entitled "**Fish Processing Waste as Potential Source of Apatite for Bone Replacement**". I also declare that it has not been previously submitted for the award of any degree or diploma or other similar title of this for any other examining body or university.

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AKNOWLEDGEMENTS

First of all, I would like to show my deepest and most sincere gratitude to my supervisor, Dr. Nurazreena Binti Ahmad. Without her constant guidance and assistance in every step throughout the process, this project would not have been accomplished. I would like to thank my co-supervisor Dr. Ku Marsilla for her encouragement and insightful comments.

I also would like to thank School of Materials and Mineral Resources University Sains Malaysia for this opportunity to conduct and complete my Final Year Research Project as a requirement to complete my Bachelor's Degree in Materials Engineering.

I am grateful to the postgraduate students for their patience and guidance in overcoming problems and challenges I have been facing through my project. I would also like to thank all the assistant engineers especially En. Mohamad Shafiq Bin Mustapa Sukri, En. Mohammad Azrul Bin Zainol Abidin and En. Abdul Rashid Bin Selamat for their professional help and cooperation.

Last but not the least, I would like to express my gratitude to my family and friends for their continuous encouragement throughout this Final Year Research project.

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LIST OF ABBREVIATIONS

BCP	Biphasic calcium phosphate
BG	Bioactive Glass
DCPD	Calcium hydrogen phosphate dihydrate
CO_3Ap	Carbonate apatite
CP	Calcium phosphate
DSC	Differential scanning calorimetry
FPH	Fish protein hydrolysate
FTIR	Fourier transform infrared spectroscopy
HA	Hydroxyapatite
ICDD	International Centre for Diffraction Data
PCL	Polycaprolactone
PDLLA	Poly-DL-lactide
PGA	Poly-lactic-glycolic acid
PLA	Poly(lactic) acid
PLGA	Poly(lactic-co-glycolic)
PMMA	Poly-methyl methacrylate
PVA	Polyvinyl alcohol
SEM	Scanning electron microscopy
TCP	Tricalcium phosphate
TGA	Thermogravimetric analysis
XRD	X-ray diffraction

LIST OF SYMBOLS

wt%	Weight percentage
%	Percent
ρ	Density
β	Beta
α	Alpha
º	Degree
ºC	Degree Celsius
θ	Theta
cm	Centimetre
μm	Micrometer
mm	Millimetre
nm	Nanometer
kPa	kiloPascal
MPa	megaPascal
GPa	gigaPascal
kN	kiloNewton

SISA TULANG IKAN SEBAGAI SUMBER APATIT UNTUK PENGGANTIAN TULANG

ABSTRAK

Dalam kajian ini, perancah berbilang fasa hydroxyapatite/β-trikalsium fosfat/karbonat apatit (HA/β-TCP/CO_3Ap) dimasukkan dengan alginat telah dihasilkan. HA/β-TCP telah diperolehi daripada pembakaran tulang ikan Tilapia (*Oreochromis mossambicus*). Sluri yang dihasilkan daripada serbuk tulang ikan Tilapia telah digunakan sebagai bahan mentah bagi proses pengeringan beku untuk menghasilkan kerangka. Perancah telah dibakar pada suhu 1200 ℃, 1300 ℃ dan 1400 ℃ selama 6 jam. Keputusan XRD menunjukkan sebahagian HA telah dijadikan β-TCP pada suhu 1200 ℃ dan 1300 ℃ manakala HA, β-TCP dan α-TCP didapatkan pada suhu 1400 ℃. Kekuatan mekanikal dan ketumpatan sampel meningkat dengan suhu pembakaran dan menurun pada suhu 1400 ℃, manakala keliangan sampel adalah sebaliknya. Sampel pada suhu 1400 ℃ telah dipilih untuk menjalakan tindak balas larutan seterusnya kerana α-TCP yang terkandung dalam kerangka dapat ditransformasikan kepada CO_3Ap dengan cepat. Transformasi kepada perancah berbilang fasa HA/β-TCP/CO_3Ap telah dijalankan dengan menggunakan tindak balas larutan-mendakan dengan 2M natrium karbonat (Na_2CO_3) pada suhu 200°C untuk 24 jam. Keputusan FTIR telah mengenalpasti kewujudan ciri-ciri kumpulan fungsian karbonat perancah berbilang fasa HA/β-TCP/CO_3Ap yang dihasilkan seterusnya dimasukkan 5 wt% natrium alginat. Keputusan FTIR dan SEM telah mengenalpasti kewujudan natrium alginate dalam perancah berbilang fasa HA/β-TCP/CO_3Ap. Kekuatan mekanikal perancah berbilang fasa HA/β-TCP/CO_3Ap bertambah baik dengan natrium alginat.

FISH BONE WASTE AS POTENTIAL SOURCE OF APATITE FOR BONE REPLACEMENT

ABSTRACT

In this research, multiphasic hydroxyapatite/β-tricalcium phosphate/carbonate apatite (HA/β-TCP/CO_3Ap) scaffolds incorporated alginate was obtained. HA/β-TCP was obtained from Tilapia (*Oreochromis mossambicus*) fish bone by thermal calcination method. Slurry made of fish bone powder was used in freeze drying method to produce porous scaffold. The porous scaffold produced was then sintered up to 1200 ℃, 1300 ℃ and 1400 ℃ for 6 hours to convert HA into β-TCP or α-TCP phase. XRD analysis revealed that partial of the HA has transformed into β-TCP at sintering temperature 1200 ℃ and 1300 ℃ whereas HA, β-TCP and α-TCP were present at 1400 ℃. Mechanical strength and density of porous scaffold increase with sintering temperature to 1300 ℃ and decrease at 1400 ℃, whereas porosity is vice versa. HA/β-TCP/α-TCP scaffold obtained from 1400 ℃ was chosen to transform into multiphasic HA/β-TCP/CO_3Ap scaffold by dissolution-precipitation reaction with 2M of sodium carbonate (Na_2CO_3) at 200 ℃ for 24 hours. Fourier transform infrared spectroscopy (FTIR) analysis confirmed the presence of characteristic functional groups of carbonate after dissolution-precipitation reaction. Multiphasic HA/β-TCP/CO_3Ap scaffold was then coated with 5 wt% of sodium alginate solution. FTIR and scanning electron microscope (SEM) analysis confirmed the presence of sodium alginate after the coating was done on the multiphasic HA/β-TCP/CO_3Ap scaffold. Mechanical strength of alginate coated multiphasic HA/β-TCP/CO_3Ap scaffold was also increased compared to that of without coating.

CHAPTER 1

INTRODUCTION

1.1 Research Background

According to the Food and Agricultural Organization (FAO, 2015), 88% (149 million tonnes) of total fishery production was used for direct human consumption. The remaining 12% (20 million tonnes) was utilised for non-food products, mainly for the manufacture of fishmeal and fish oil. It is estimated that more than 50% of the remaining material from the total fish capture is processing waste and not used as food (Sila & Bougatef, 2016), creating both disposal and pollution problems. Fish waste management has been one of the problems that lead to environmental hazards.

Instead of merely managing the issue of waste disposal, considerable research effort has been directed towards the conversion of fish wastes into useful products. The major fish waste from fish processing industry, which is approximately 70% of the original raw material, include bone frames, bones, viscera, head, skin, and scales (Sila & Bougatef, 2016). Majority of fish wastes are used to produce fish oil, fishmeal, fertilizer, animal feed and fish silage (Malaweera & Wijesundara, 2014). The skin and bone of fish have high collagen and gelatin content which are used in food, pharmaceuticals, and cosmetics industries (Olsen *et al.*, 2014). Besides, fish waste such as internal organs have a high content of a variety of enzymes which is useful in the field of biotechnology and biochemistry (Fernandes, 2016). The amount of fish bone fraction, which is about 10- 15% of the total body weight, is regarded as a waste product (Nemati *et al.*, 2017). Fish bone derived hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) has been studied widely for orthopaedic and dental applications.

HA has excellent biocompatibility, bioactivity and osteoconductivity, hence HA is widely used as a biomaterial in many biomedical applications (Wu *et al.*, 2016). HA can also be extracted from natural resources. It is the main component of animal and fish bones, which accounts approximately 60 - 70% of their weight while the remaining part of the bone consists of organic molecules, mainly collagen and a variety of noncollagenous proteins (Herpandi *et al.*, 2011). For instance, Piccirillo et al. (2013) has successfully extracted HA and tricalcium phosphate (TCP) from codfish bones. Sunil and Jagannatham (2016) have successfully produced pure HA from South Indian River fish bones by simple heat treatment.

HA, TCP and biphasic calcium phosphate (BCP) are calcium phosphate (CP) compounds that have been used as bone substitutes due to the similarity in chemical composition to native bone (Ebrahimi & Botelho, 2017). Often researchers produce HA from several synthetic methods and techniques, such as solid-state reaction, sol–gel and hydrothermal processing. The properties of synthetic HA depend on its particle size, morphology, crystallinity, and composition, which require strict control of synthesis precursors and processing conditions (Dasgupta *et al.*, 2013).

Human bone mineral has always been idealised as HA, but it actually differs in composition from stoichiometric HA. The inorganic component of human bone is carbonate apatite (CO_3Ap), which contains up to 8 wt% of carbonate with multisubstituted ions (Na^+, Mg^{2+}, K^+, F^-, Cl^-, etc.) in the apatite structure (Bang *et al.*, 2015; Tsuru *et al.*, 2017). The carbonate ion substitutes either at the hydroxyl site or at the phosphate tetrahedron, giving A- and B-type CO_3Ap, respectively. The presence of carbonate in the apatite lattice lowers the stability of the apatite structure which contributes to the ease of resorption in bone tissue. $Ca–CO_3^{2-}$ bonds being weaker than the $Ca–PO_4^{3-}$ bonds leads to the higher solubility of carbonate-containing

apatite compared to stoichiometric HA (Šupová, 2015). CO_3Ap would be a better candidate as a bone scaffold due to its high osteoconductivity and ability to be replaced by new bone (Sugiura *et al.*, 2017).

It is believed that CO_3Ap could be used for design of scaffold enabling growth of damaged bone. Scaffold provides a temporal structural support for bone growth during regeneration of the tissue. Bone scaffold must be biocompatible to enable cell adhesion, differentiation, and proliferation (Elamparithi & Moorthy, 2017). The material should induce strong bone bonding besides having the biodegradability at an appropriate rate to new tissue formation (Bose *et al.*, 2012). Scaffold must be osteoconductive to allow the growth of host bone into the scaffold (Liu *et al.*, 2013). Also, bone scaffold should have sufficient mechanical stability to provide a suitable environment for new bone tissue formation (Elamparithi & Moorthy, 2017). In addition, an ideal scaffold must have interconnected porous structure with porosity of 50% and pore size greater than 100-200 μm for cell penetration and tissue in growth (Liu *et al.*, 2013; Thavornyutikarn *et al.*, 2014; Zubairi *et al.*, 2016).

However, it is known that mechanical strength is governed by the porosity. High porosity results in low mechanical strength and vice versa. To overcome this disadvantage, brittle scaffolds are coated with a bioresorbable polymer layer to fill the microcracks in the bioceramic structure (Yong *et al.*, 2018). Polymer filaments will induce crack-bridging mechanisms during fracture thus increasing the fracture toughness (Philippart *et al.*, 2015). Alginate is used as the polymer coating on the scaffold in this research due to its abundant sources, biocompatibility and low toxicity (Lee & Mooney, 2012). Alginate has been employed as a hydrogel due to its hydrophilic nature, which is beneficial to minimize the protein adsorption and cell adhesion (Yong *et al.*, 2018).

1.2 Problem Statement

The production of HA by synthetic methods are complicated, time consuming and expensive which creates limitations in producing it in large quantities (Kang *et al.*, 2013). HA obtained from natural resources have greater advantages compared to synthetic one. Fish wastes like fish bone is a composite material made of carbonated HA, type I collagen, non-collagenous protein and water. Biological apatite from fish bone has chemical composition similar to that of human bone mineral which contains CO_3^{2-} group and various amount of trace elements such as Na^+ and Mg^{2+} (Naga *et al.*, 2015; Wu *et al.*, 2016). Fish bone derived HA has better biocompatibility and bioactivity compared to synthetic one due to the disordered nanostructures and nonstoichiometric composition (Boutinguiza *et al.*, 2012). Moreover, the production of HA from fish bone waste is more economical and environmentally friendly.

Nevertheless, carbonate groups in carbonated HA will be eliminated by decomposing into carbon dioxide at high temperature required for sintering process (Boutinguiza *et al.*, 2012; Suguira *et al.*, 2017; Tsuru *et al.*, 2017), which in turn affects the biological properties of the apatite obtained by thermal calcination method (Venkatesan *et al.*, 2011). Research study has demonstrated that apatite containing carbonate ions can be fabricated based on dissolution-precipitation reactions using a synthetic precursor (Nagai *et al.*, 2015). The precursor must contain at least one component of CO_3Ap such as calcium, phosphate or carbonate. It also should be in a metastable phase and have low solubility in the solution (Nomura *et al.*, 2014). For example, α-tricalcium phosphate (TCP, (α-$Ca_3(PO_4)_2$)) can be used as a precursor as it has suitable solubility and is stable under sintering temperature (Tsuru *et al.*, 2017).

Considering the advantages of natural HA, fish bones are used as the source of natural HA in this project. Porous scaffold will be produced by the freeze casting

method followed by sintering. The stability of HA phase in the fish bone is dependent on the sintering temperature. Heat treatment above 950 ℃ on fish bone eliminates carbonate content (Boutinguiza *et al.*, 2012) while sintering at high temperatures ~1400 ℃ eliminates the functional group OH in the HA matrix and results in the decomposition of HA with the formation of TCP phase (Wen *et al.*, 2016). In order to obtain carbonate content that is lost during heat treatment, unstable TCP can be transformed to CO_3Ap in carbonate solutions (Ishikawa *et al.*, 2018) by dissolution-precipitation reaction. Therefore, CO_3Ap will be fabricated using dissolution–precipitation and precursors with unstable phase α-TCP. Finally, a polymer layer using alginate will be used to coat the CO_3Ap scaffolds to improve the mechanical properties.

1.3 Objectives

1. To evaluate the effect of sintering temperature on the fish bone waste.
2. To evaluate the feasibility of CO_3Ap formation from fish bone waste via dissolution-precipitation method.

1.4 Thesis Outline

Chapter 1 provides the overview of the research and a brief literature survey of the previous and relevant work. It discusses on the introduction of dissertation including background of fish processing waste, apatite and tissue engineering, problem statement, objectives of this study and the scope of this project.

Chapter 2 highlights the detailed literature review of previous and relevant works about fish bone waste, natural resources and synthetic route for the synthesis of

HA, the type of materials for bone replacement, the fabrication methods of porous scaffolds for bone tissue engineering, and the fabrication of carbonate apatite.

Chapter 3 presents the raw materials, methodology and the characterization techniques used in this project. Chapter 4 gives the interpretation and discussion of the experimental results of the research work. Chapter 5 presents the significance of the results and the summary of the research study. The recommendations for the future studies in the related research are included in this chapter.

CHAPTER 2

LITERATURE REVIEW

2.1 Fish Processing Waste

Worldwide about 970–2700 billion tons of fish are caught, among which 450–1000 billion are used for human consumption, fish oil extraction and the remaining is a waste (Fishcount.org.uk, 2018). As a result, every year a considerable amount of total catch is discarded as processing waste which includes trimmings, fins, frames, heads, skin and viscera. Their percentage can vary between 50% and 70% of the fresh weight according to each species (Villamil *et al.*, 2017). Table 2.1 shows the average composition of fish waste.

Table 2.1 Average proportion of fish waste (Gálvez & Bergé, 2013).

Component	Average Weight (%)
Head	9-20
Backbone	9-15
Viscera	12-18
Skin	1-3
Trimmings	8-17

Ghaly et al. (2013) explained the steps of fish processing in the industry which involves stunning, grading, removal of slime, scaling, washing, deheading, gutting, cutting of fins, slicing into steaks, filleting and meatbone separation. The first step is

stunning which produces movements to rupture the bones and blood vessel. The next step is grading where fish is sorted by species and size, followed by slime removal through continuous washing. Next, scaling is carried out to remove the scales from the fish. The fish will then be washed to clean and remove the accumulated bacteria on the fish. The inedible fish head and internal organs as well as fins will be removed during deheading, gutting and fins cutting steps. Finally, filleting step will be carried out to get trimmed fillet and the meat will be removed from skin, scales and bones during process of meat bone separation. Figure 2.1 illustrates the fish processing steps in the fish processing industry.

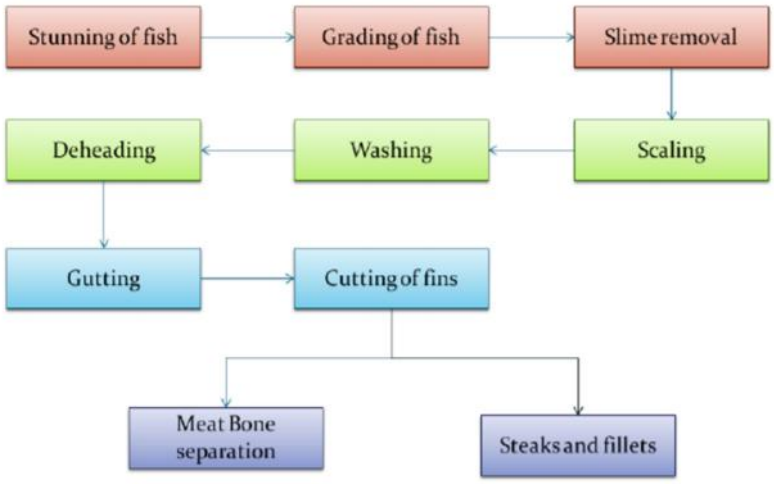

Figure 2.1 Fish processing steps (Ghaly *et al.*, 2013).

In these operations, fish wastes such as head, bone, tails, skin, gut, fins and frames as well as water and oils were generated. It was also reported by Okereke & Onunkwo (2014) that deheading, gutting and deboning as well as washing process in the production of fish crackers generated wastes such as bones, guts and fins as well as huge volume of waste effluents. Ineffective management of waste leads to burdensome disposal problems and environmental concerns especially waste organic

odor problem (Bin *et al.*, 2013). Therefore, an integrated and sustainable utilisation of fisheries resources is necessary to reduce the environmental issues and support sustainable environmental development.

Fish waste such as heads, viscera, trimmings, fish bones or cartilage, tails, and eggs can be converted into useful products as shown in Table 2.2. They contain various valuable compounds such as calcium and phosphorus, lipid and protein with long-chain omega-3 fatty acids, micronutrients and others (Sila & Bougatef, 2016). Fish viscera have both high protein and lipid content whose variability in composition depends on the species, season, age, sex, and other factors (Villamile *et al.*, 2017). Collagen and gelatin in bone and skin can be used as an alternative to mammalian collagen for food and pharmaceutical applications (Jayathilakan *et al.*, 2012). Kittiphattanabawon et al. (2005) extracted collagens from bigeye snapper (*Priacanthus Tayenus*) fish bones and type I collagen with slightly different amino acid compositions were identified.

There is an intensive attempt for the development and extraction of HA from fish bone waste for reconstructive bone replacement and other medical applications due to low production cost and excellent biocompatibility (Bin *et al.*, 2013; Naga *et al.*, 2015; Yamamura *et al.*, 2018). Fish bone is a made of carbonated HA, type I collagen, non-collagenous protein and water (Pal *et al.*, 2017). HA can be used as implant materials in bone replacement, heart valves, hip extension and other implants in the human body due to the similar chemical composition to human bones (Bin *et al.*, 2013). This will be further discussed in section 2.5.

Table 2.2 Potential uses of fish waste.

Fish Waste	Valuable Compounds	Uses
Skin	Collagen, gelatin	Food, cosmetic, and biomedical industries (Sila *et al.*, 2015).
Scale	Collagen, HA	Cosmetic and biomedical industries (Paul *et al.*, 2017).
Oil	Unsaturated fatty acids	Biodiesel production (Saifuddin & Boyce, 2017), pharmaceutical and food industry (Olsen *et al.*, 2014).
Bone	Calcium, HA, collagen	Biomedical applications (Akram *et al.*, 2014).
Internal Organs	Enzymes	Biotechnology and biochemistry industries (Fernandes, 2016).

2.2 Basics of Human Bone Structure

Human bone is a complex organic–inorganic nanocomposite structure. It is made up of an organic phase with mainly type I collagen, a mineral phase of carbonated apatite and lastly other non-collagenous proteins that form a microenvironment stimulatory to cellular functions (Henkel *et al.*, 2013). The collagen is responsible for the bone's rigidity, viscoelasticity and toughness while the inorganic phase of carbonated apatite crystals is responsible for structural reinforcement, stiffness and mineral homeostasis (Henkel *et al.*, 2013; Wang *et al.*, 2014; Pasteris, 2016).

Human bone exhibits a hierarchical structure as illustrated in Figure 2.2, which include the macrostructure (cancellous and cortical bone), the microstructure (haversian systems, osteons, single trabeculae - 10 to 500 μm), the sub-microstructure (lamellae - 1 to10 μm), the nanostructure (fibrillar collagen and embedded mineral - few hundred nm to 1 μm) and the subnanostructure (mineral, collagen, and non-collagenous organic proteins - below few hundred nm) (Henkel *et al.*, 2013; Tejero *et al.*, 2014; Wang *et al.*, 2016).

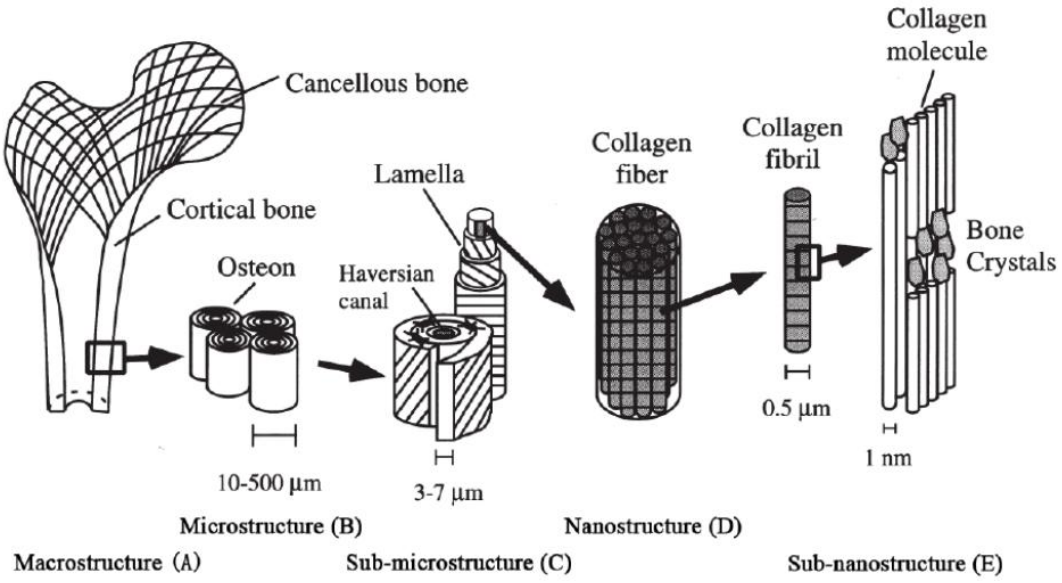

Figure 2.2 Hierarchical structural organization of bone: (A) cortical and cancellous bone; (B) osteons with Haversian systems; (C) lamellae; (D) collagen fibre assemblies of collagen fibrils; (E) bone mineral crystals, collagen molecules, and non-collagenous proteins (Henkel *et al.*, 2013).

Bone has two architectural forms which are cortical bone and cancellous bone (Figure 2.3). Bone surfaces consist of cortical bone, which is a very compact nanocomposites (3-5% porosity), and the thickness of this external hard tissue increases in mechanically demanding regions such as long bones (Pasteris, 2016;

Wang *et al.*, 2016). Cancellous bone has a porosity of 50 - 90% and the pores are filled with bone marrow. It is found in the bone interior, such as within the vertebra (Pasteris, 2016; Wang *et al.*, 2016). The mechanical properties of bone and bone components are shown in Table 2.3. The mechanical properties depend on the bone composition including porosity and mineralisation, as well as the structural organisation such as trabecular or cortical bone architecture and collagen fibre orientation.

Table 2.3 Mechanical properties of bone and bone components (Henkel *et al.*, 2013; Pasteris, 2016; Wang *et al.*, 2016).

Properties	Compressive Strength (MPa)	Tensile Strength (MPa)	Young's Modulus (GPa)
Cortical Bone	100-230	50-150	7-30
Cancellous Bone	2-12	10-20	0.5-0.05
Collagen	-	100	1.5
Hydroxyapatite	-	9-120	80-117

The main inorganic crystal phase within bone is called biological apatite. As shown in Table 2.4, bone mineral or biological apatite is CP apatite containing up to 8 wt% CO_3^{2-} ions and traces of Na^+, Mg^{2+}, Fe^{2+}, Cl^-, F^- and other ions which localized interstitially and substitutionaly into its structure (Bang *et al.*, 2015). It is known as carbonate apatite (CO_3Ap, $(Ca_{10-a}(PO_4)_{6-b}(CO_3)_b(OH)_{2-c})$), where a=substituent for Ca, such as Mg, Na, Sr, etc; c=substituent for OH such as F or Cl. Bone mineral has Ca/P ratios ranging from 1.37 - 1.87.

Carbonate groups in bone minerals can be found in A-type (OH-) or B-type (PO_4^{3-}) based on the substitution site of carbonate ions. B-type CO_3Ap is found to be more similar in structure to human bone (Tsuru *et al.*, 2017). The carbonated and hydrated phase that constitutes bone apatite have different composition from HA, which accounts for the higher solubility and smaller crystallite size of bone apatite compared to that of stoichiometric HA (Pasteris, 2016). B-type carbonate substitution causes decreases in *a*-axial length together with increases in *c*-axial length, changes in crystallite size, and changes in the amount of crystallographic microstrain and mechanical strengthening of the bone. The increase in solubility is due to the weaker Ca–CO_3 bonds than Ca–PO_4 bonds (Šupová, 2015).

Table 2.4 Composition of inorganic phases of human bone (Tsuru *et al.*, 2017).

Components	Composition (wt %)
Calcium, Ca^{2+}	34.8
Phosphorus, P	15.2
Sodium, Na^{+}	1.71
Magnesium, Mg^{2+}	0.72
Potassium, K^{+}	0.03
Carbonate, CO_3^{2-}	7.4
Fluoride, F^{-}	0.03
Chloride, Cl^{-}	0.13
Pyrophosphate, $P_2O_7^{4-}$	0.07
Absorbed H_2O	10.0

2.3 Bone Substitute Materials

Bone is the second most common transplant tissue after blood. Bone replacement materials are needed for the production of bone implants and to repair bone defects. A bone substitute is defined as "a synthetic, inorganic or biologically organic combination which can be inserted for the treatment of a bone defect instead of autogenous or allogenous bone" (Campana *et al.*, 2014). Ideally, a bone substitute should be mechanically strong as cortical or cancellous bone being replaced, resorbable, and osteoinductive (Bohner *et al.*, 2012). Bone substitute materials can be categorized based on the origin of the material which includes autografts, allografts, xenografts and synthetic materials. Synthetic bone substitute materials may consist of metals, polymers, and ceramics.

2.3.1 Natural Bone Grafts

The autograft has been considered as the standard of bone graft replacements. Autogenous grafts are transferred from an individual using either extraoral sites or intraoral donor sites. Autogenous grafts are osteoconductive and support the ingrowth of new tissues and cells. They may contain osteoinductive proteins which promote the proliferation and differentiation into osteogenic cells. Osteogenic cells that hold viable cells that can form new bone tissues are also present in autogenous bone. However, autograft has limitation such as infection risk at the bone collection site, requisite of additional surgery and limited availability of fresh bone (Wang & Yeung, 2017).

Allografts are obtained from other individuals of the same species. The use of allograft bones has advantages of elimination of the risk of donor site morbidity and

the shorter surgical time. Disadvantages of allografts include the slower incorporation and less consistent clinical results compared with autografts (Ganz *et al.*, 2015). Besides, they are risks of disease transmission and immunogenic response in allografts (Wang & Yeung, 2017).

Xenogeneic graft is taken from a donor of another species, commonly bovine bone. Even though xenografts are osteoconductive, they are generally not considered for use as a bone graft substitute. They do not have osteogenic and osteoinductive properties and they may cause an immune reaction (Wang & Yeung, 2017).

2.3.2 Synthetic Bone Substitutes Materials

Owing to the combination of high strength and toughness, metals have been widely used for major load-bearing orthopaedic applications. Metallic biomaterials such as stainless steels, titanium and cobalt–chromium-based alloy are commonly used. Shortcomings of these metallic materials include the release of toxic metallic elements in the human body due to corrosion and wear (Gu *et al.*, 2014). Moreover, use of metallic material may result in bone loss due to stress shielding since metal has higher stiffness than bone (Matassi *et al.*, 2013). Even though magnesium and its alloy has been extensively studied and developed as metallic biomaterial, rapid degradation in physiological environment and local corrosion mechanism cause the loss of mechanical integrity before complete healing of tissue and also hydrogen accumulation and localized alkalization (Chen *et al.*, 2014; Gu *et al.*, 2014; Gao *et al.*, 2017).

Polymers have also been widely used for bone repair and tissue engineering due to the biodegradability, controlled degradation rates (Sheikh *et al.*, 2015), non-

corrosion properties and also flexibility in processing (Bose *et al.*, 2012). Some of the polymers commonly used include poly(lactic acid) (PLA), poly-lactic-glycolic acid (PGA), and poly-methyl methacrylate (PMMA). However, the polymeric materials show rapid strength degradation *in vivo* (Bose *et al.*, 2012). Degradation of polymers such as PLA, PGA creates a local acidic environment that can hinder the cell growth on bone biomaterials and induce inflammatory reaction (Sheikh *et al.*, 2015; Gao *et al.*, 2017). Besides, lower stiffness of polymeric implants than that of natural bone results in easy deformation of the implants during *in vivo* use (Zhao, 2011). The monomer, MMA, from PMMA which is considered an irritant and a potential carcinogen, might be released into the physiological system and cause a drastic fall in pressure (Bose *et al.*, 2017).

Bioceramics such as bioactive glass (BG) and calcium phosphates (CP) have been widely studied for bone replacement applications. BG consists of calcium, phosphate, and silicate as the main component. The composition of the components can be varied thus they can be used both in hard and soft-tissue healing applications (Fiume *et al.*, 2018). The conventional silicate glass 45S5 bioactive glass (molar ratio: 46.1% SiO_2, 24.4% Na_2O, 26.9% CaO, and 2.6% P_2O_5) is commonly used as bone graft substitutes. However, it has poor sinterability, which resulted in scaffolds with low strength. Dense network of porous scaffold is difficult to be achieved due to the limited ability of 45S5 glass to sinter by viscous flow above its glass transition temperature. Besides, slow degradation rate and incomplete conversion of the scaffold to an HA-like material result in the mismatch of the degradation rate of the scaffold with the new tissue formation rate (Rahaman *et al.*, 2011).

CP materials are drawing a great attention due to the excellent biocompatibility, bone bonding ability and compositional similarity to the bone (Bang *et al.*, 2015).

Most of the CP used for bone substitutes are based on HA, β-TCP, BCP, which is a mixture of β-TCP and HA (Wang *et al.,* 2014). However, stoichiometric HA has low resorption rate and remains at the implant site for a long time after implantation (Bang *et al.*, 2015; Sheikh *et al.*, 2015). In fact, as discussed earlier, the bone mineral is carbonated HA known as CO_3Ap. Therefore, CO_3Ap would be a better material than stoichiometric HA for bone substitute which requires replacement by natural bone and hard tissues. β-TCP has lower Ca/P ratio of 1.5 and thus more degradable and becomes soluble more rapidly (Wang & Yeung, 2017). In addition, the released calcium and phosphate ions from β-TCP induce bone regeneration (He *et al.*, 2017). BCP which is composed of a stable phase, HA and a more soluble phase, β-TCP, present several advantages over the single-phase components or other CP materials owing to their better dissolution rate accelerating new bone formation (Villalá *et al.*, 2017).

2.4 Biphasic Beta-Tricalcium Phosphate/Carbonate Apatite

During the bone remodeling process, osteoclasts produce a weak acidic environment in Howship's lacuna at pH 3–5 to dissolve bone minerals. In this case, HA is stable and has very low solubility compared to β-TCP, which impedes the regeneration of bone (Sunouchi *et al.*, 2012; Dorozhkin, 2015; Ruddyard *et al.*, 2017). β-TCP has good osteoconductivity and biocompatibility. A research done by Lu et al. (2002) proved that a new lamellar bone was formed following direct contact with the β-TCP implant and surrounding osteoblasts after a few weeks of implantation. However, β-TCP presents poor reactivity as a pure material due to the slow formation in vivo bone formation and less-than-ideal degradation or solubility (Xie *et al.*, 2016).

In the 1970s and 1980s, sintered HA was widely used as an artificial bone substitute. However, high crystallinity and the loss of carbonate of the sintered HA result in a much lower rate of biodegradation compared to the nanocrystalline bone mineral (Bohner *et al.*, 2012). Solubility of apatite under weak acidic conditions increases with carbonate content in its apatitic structure (Sunouchi *et al.*, 2012; Nomura *et al*., 2014). Carbonate substitution decreases the crystallinity and increases the solubility and chemical reactivity due to the weak bonding (Ana *et al.*, 2010). This can be supported by a recent study done by Bang et al. (2015) where the introduction of carbonate ions in the HA lattice caused a decrease in the crystallinity of the HA and promoted better bioactivity of the sintered body. Thus, CO_3Ap is soluble in Howship's lacuna whereas HA is not. In addition, CO_3Ap increases the local concentration of calcium and phosphate ions for new bone formation. CO_3Ap can upregulate differentiation of osteoblasts and demonstrated higher osteoconductivity than sintered HA. (Bang *et al.*, 2014; Mohammad *et al.*, 2015; Nakamura *et al.*, 2016; Tsuru *et al.*, 2017).

2.4.1 Fabrication of Carbonate Apatite

Sintering is commonly used to produce bioceramics with the required mechanical properties by the consolidation of powder compact. However, sintering methods cannot be used for the fabrication of CO_3Ap bone substitute. Doi et al. (1993) reported that the carbonate is liberated as carbon dioxide gas from CO_3Ap at 600 ℃ to 950 ℃. Thermally decomposed CO_3Ap showed alkalinity, and thus results in a much lower rate of biodegradation compared to the nanocrystalline bone mineral. Moreover, cytotoxicity was shown when the amount of liberated carbon dioxide is high.

Phase transformation of unstable precursors based on dissolution-precipitation reactions has been used for the fabrication of CO_3Ap bioceramics. Calcite ($CaCO_3$) (Wakae *et al.*, 2008), gypsum ($CaSO_4 \cdot 2H_2O$) (Nomura et al., 2014), and α-TCP (Sugiura *et al.*, 2016; Mamat *et al.*, 2017) are commonly used precursors. There are requirements for the precursor involved in the fabrication of CO_3Ap block. First, a precursor should be a block. Compositional transformation from the precursor to CO_3Ap block occurs through a dissolution–precipitation reaction, maintaining the macroscopic structure of the precursor. Second, the precursor should contain at least one of the elements of CO_3Ap such as calcium, phosphate or carbonate (Tsuru *et al.*, 2017). Third, the precursor should be in a metastable phase and show proper solubility (Maruta *et al.*, 2011; Tsuru *et al.*, 2017). The precursor phase dissolves in aqueous solution and releases ions that are required for the precipitation of the CO_3Ap. For example, Ca^{2+} and CO_3^{2-} released from $CaCO_3$ combine with the PO_4^{3-} ions in phosphate solution produces CO_3Ap (Wakae *et al.*, 2008). The precursor TCP supplies Ca^{2+} and PO_4^{3-} and precipitation occurs on the surface of TCP as CO_3Ap in carbonate solution according to Equation 2.1 and 2.2 (Sugiura *et al.*, 2016; Mamat *et al.*, 2017), respectively.

$$Ca_3(PO_4)_2 \rightarrow 3Ca^{2+} + 2PO_4^{3-} \quad (2.1)$$

$$Ca^{2+} + PO_4^{3-} + CO_3^{2-} \rightarrow Ca_{10-a}(PO_4)_{6-b}(CO_3)_c(OH)_{2-d} \quad (2.2)$$

Tsuru et al. (2017) used calcium hydrogen phosphate dihydrate (DCPD) block as a precursor to fabricate CO_3Ap block by immersing it in $NaHCO_3$ or Na_2CO_3 solution. Mamat et al. (2017) reported that CO_3Ap can be fabricated using a

β-TCP scaffold precursor from freeze drying method and immersed it into 5M of Na_2CO_3 solution. The results showed that 11 wt% of carbonate content was obtained from hydrothermal treatment at 200°C for 5 days. In a more recent study carried out by Yong et al (2018), biphasic β-TCP/α-TCP scaffold fabricated from polymer reticulate method was used as a precursor. Biphasic β-TCP/CO_3Ap scaffold was produced by dissolution-precipitation reaction in 1M of $NaHCO_3$ at 170°C for 5 days.

2.5 Sources of Hydroxyapatite

The origin of HA ceramic may be natural or synthetic. Synthetic methods such as solid-state reaction, wet chemical precipitation and sol gel method (Kaygili & Keser, 2015; Sopyan *et al.*, 2017) have been developed to produce HA. HA can also be produced by natural sources which include sea corals (Adnen *et al.*, 2017), egg shells (Wu *et al.*, 2015; Ramesh *et al.*, 2016), fish bones (Pal *et al.*, 2017) and bovine bones (Niakan *et al.*, 2015; Bano *et al.*, 2017).

2.5.1 Synthetic Routes

Wet chemical precipitation and sol–gel methods are some of the popular techniques to synthesis HA powder. For wet chemical precipitation, large amount of HA powder can be produced without organic solvents at a reasonable cost. However, special pH value control condition is required because the degree of neutralization of phosphoric acid is pH dependent (Ramesh *et al.*, 2015; Wu *et al.*, 2015). It is rather time consuming and complicated (Ho et al., 2013; Wu *et al.*, 2015). Meanwhile, due to the slow reaction between calcium and phosphorus precursors in the sol phase, sol–

gel synthesis requires a strict control of process parameters and an intimate contact molecular mixing of calcium and phosphorous (Ramesh *et al.*, 2015).

Stoichiometric HA prepared from synthetic methods do not contain trace amounts of ions in its lattice structure (Kamalanathan *et al.*, 2014). Moreover, the properties of synthetic HA depend on its particle size, morphology, crystallinity, and composition, which require suitable synthesis precursors and processing conditions (Wu *et al.*, 2016). The synthetic production of HA often requires the use of hazardous chemicals, ageing processes and an imbalanced stoichiometric ratio (Venkatesan *et al.*, 2015).

2.5.2 Biological Source

HA extracted from natural resources is non-stoichiometric which contain various trace amounts of ions such as CO_3^{2-}, Na^+, Mg^+ and Sr^- (Akram *et al.*, 2014; Wu *et al.*, 2015). The presence of these ions plays an important role in bone regeneration.

Eggshells have been extensively studied to synthesis HA powder. An eggshell contains calcium carbonate, magnesium carbonate, calcium phosphate, and organic matter, together with some trace elements, such as K, Na, Mg, and Sr, which are found in human bone (Wu *et al.*, 2013; Ramesh *et al.*, 2016). Wet chemical precipitation method (Kamalanathan *et al.*, 2014), hydrothermal method (Wu *et al.*, 2013), wet mechanochemical method (Gergely *et al.*, 2010) and microwave irradiation method (Krishna *et al.*, 2007) have been adopted to obtain HA from eggshells. More recently, Ramesh et al. (2016) employed direct solid-state sintering method to synthesis nanostructured HA from eggshell.

Considerable research has been done on extraction of HA from seashells due to the wide availability and low cost. Trinkunaite-Felsen et al. (2016) fabricated high purity crystalline HA from seashells using water-based sol-gel synthesis route. Ge et al. (2016) synthesised carbonate substituted HA through one-step hydrothermal exchange from seashells. Zubir et al. (2015) reported that aragonite phase present in the corals can be transformed to HA via hydrothermal treatment. More recently, Adnen et al. (2017) studied the effect of calcination temperature towards properties of HA obtained from corals. However, corals have a slow growth rate and are in danger of extinction (Ho *et al.*, 2013; Wu *et al.*, 2015).

Significant efforts have been made to synthesis natural HA from bovine bone. Bovine bone is mainly composed of organic and mineral component of HA with a low percentage of other minerals such as CO_3, Mg and Na (Niakan *et al.*, 2015). Bovine bone derived HA has similar morphology and structure to human bone with features of well-defined porous structure, which is suitable for biomedical application (Niakan *et al.*, 2015). In addition, Bano et al. (2017) reported that HA extracted from bovine bone through hydrothermal followed by calcination is useful as a biomaterial in orthopaedic and dental implants. However, bovine origins are often associated with disease transmission and religious sentiment (Venkatesan *et al.*, 2015).

On the other hand, fish sources are presumably much safer and exhibit a low risk of disease transmission due to the wide evolutionary gap between fish and humans (Venkatesan *et al.*, 2015). Fish bones are also a good source of calcium, phosphate, and carbonate which can be used to prepare HA. For example, Piccirillo et al. (2013) has successfully produced calcium phosphate biphasic material consisting of HA and β-TCP by annealing codfish bones. According to the study reported by Sunil and Jagannatham (2016), pure HA could be produced from Sheelavati fish

bones by a simple heat treatment. The *in vitro* studies done by Pal et al. (2017) reported the non-toxic nature of fish bone derived HA. Shi et al. (2018) confirmed the presence of CO_3^{2-} and Mg^{2+} in HA derived from rainbow trout and salmon bones which are useful as bone implant material substitute. Thus, fish bones could be a promising raw material for biomedical applications.

2.6 Scaffolds in Bone Tissue Engineering

Tissue engineering is defined as a multidisciplinary scientific branch that combines cell biology, materials science and engineering, and regenerative medicine (Thavornyutikarn *et al.*, 2014). In bone tissue engineering, a scaffold acts as the matrix that serves as a host for tissue formation. Design and fabrication of the 3D scaffolds is of critical importance. An ideal scaffold should have an interconnected porous structure with high porosity to support cell infiltration and vascularization and also to allow diffusion of waste products out of the scaffold (Liu *et al.*, 2013; Thavornyutikarn *et al.*, 2014; Zubairi *et al.*, 2016). It should have specific surface properties to allow the adhesion of the cell tissues, differentiation and proliferation of the cells (Thavornyutikarn *et al.*, 2014; Siva & Ansari, 2015; Zubairi *et al.*, 2016). Adequate mechanical strength is also required to maintain the predetermined tissue structure and biocompatibility (Elamparithi & Moorthy, 2017). The specific criteria of an ideal scaffold in bone tissue engineering are summarised in Table 2.5.

Table 2.5 Criteria of an ideal scaffold for bone tissue engineering (Liu *et al.*, 2013; Thavornyutikarn *et al.*, 2014; Siva & Ansari, 2015; Zubairi *et al.*, 2016).

Criteria	Requirement
Biocompatibility	Support and foster cell attachment, proliferation, and differentiation.
Biodegradability	Degrade at rates appropriate to tissue regeneration.
Osteoconductivity	Encourage host bone adherence and growth into the scaffold.
Mechanical properties	Be able to maintain the structure and to withstand in vivo loading forces.
Porous structure	Interconnected with high porosity (>50%) and pore diameters between 100 and 200 μm, to support cell infiltration and vascularization and to deliver nutrients.
Fabrication	Can be produced into irregular shapes of scaffolds that match the defects in the bone.
Commercialisation	Be synthesised and fabricated at a reasonable cost for commercialisation.

2.6.1 Fabrication Techniques of Porous Scaffolds

The main fabrication techniques in fabricating porous scaffolds include polymer reticulate method, solvent casting and freeze drying. The polymer reticulate technique (Figure 2.3) involves the infiltration of a synthetic or natural template with a ceramic suspension. After drying, the template is removed thus a replica of the original template structure is formed (Studart *et al.*, 2006). High porosities up to 95% and controlled pore size of 150-200 μm can be achieved by this method. The high