SCHOOL OF MATERIALS AND MINERAL RESOURCES ENGINEERING
UNIVERSITI SAINS MALAYSIA

DEVELOPMENT OF CARBONATED HYDROXYAPATITE (CHA) POROUS SCAFFOLDS FOR BONE TISSUE ENGINEERING APPLICATIONS

By

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DECLARATION

I hereby declare that I have conducted, completed the research work and written the dissertation entitled "**Development of Carbonated Hydroxyapatite (CHA) Porous Scaffolds for Bone Tissue Engineering Applications**". 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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LIST OF ABBREVATIONS

BCP	Biphasic Calcium Phosphates
BTE	Bone Tissue Engineering
CaP	Calcium Phosphates
CHA	Carbonated Hydroxyapatite
EDX	Energy Dispersive X-Ray
FTIR	Fourier Transform Infrared Spectroscopy
HA	Hydroxyapatite
KBr	Potassium Bromide
MMSCs	Mesenchymal Stem Cells
PEG	Polyethylene Glycol
PU	Polyurethane
PVA	Polyvinyl Alcohol
SBD	Segmental Bone Defect
SBF	Simulated Body Fluid
SEM	Scanning Electron Microscopy
TCP	Tricalcium Phosphate
TE	Tissue Engineering
TEM	Transmission Electron Microscopy
XRD	X-Ray Diffraction
XRF	X-Ray Fluorescence

LIST OF SYMBOLS

°	Degree
μm	Micrometre
Å	Angstrom
℃	Degree celcius
D_v	Volume weighted crystallite size
k	Scherrer constant
MPa	Megapascal
ppi	Pores per inch
ppm	Parts per million
wt. %	Weight percentage
β_{hkl}	Line broadening at half the maximum intensity
θ	Thetha
θ_{hkl}	Bragg diffraction angle

PEMBANGUNAN KERANGKA BERLIANG HIDROKSIAPATIT BERKARBONAT UNTUK APLIKASI KEJURUTERAAN TISU TULANG

ABSTRAK

Dalam kajian ini, hidroksiapatit berkarbonat (CHA) disintesis menggunakan dua kaedah pengemulsian nano dan diikuti dengan pencirian fiziko-kimia. Serbuk CHA yang telah disintesis dalam kajian ini disahkan sebagai CHA jenis B dengan kekurangan kalsium. Kerangka CHA yang bersambungan liang telah dihasilkan dengan cara replika busa poliuretana di peringkat kedua kajian ini. Kajian ini adalah pengoptimuman rangkaian dalaman kerangaka CHA dengan memanipulasi jenis serbuk CHA yang digunakan, komposisi pengikat dan penambahan kaolin untuk memperbaiki senibina kerangka. Kerangka tersebut kemudian disinter pada 800℃ dengan dua kadar pemanasan yang berbeza iaitu 5℃/minit dan 10℃/minit selama 2 jam. Kerangka CHA dengan 5% berat kaolin dengan nisbah pengikat PEG: PVA = 2:3 yang disinter pada 800℃ dengan 5℃/minit kadar pemanasan memiliki senibina yang paling memuaskan. Kerangka CHA optimum mempunyai keliangan sebanyak 80.11% dengan 200-400 μm liang makro, topang yang berketebalan sekata dengan purata 30-50 μm, dengan rekahan yang minium dan kekuatan mampatan sebanyak 0.072 MPa. Dapat disimpulkan bahawa kadar pemanasan yang digunakan menunjukkan impak yang paling kurang dalam fabrikasi kerangka berliang CHA yang man kunci kejayaan fabrikasi kerangka berliang CHA dalam kajian ini banyak bergantung kepada komposisi buburan CHA yang digunakan. Dari segi bioaktif, kerangka kaolin CHA membentuk lapisan apatit pada permukaan kerangka selepas direndamkan dalam SBF selama 7 hari. Ini menunjukkan bahawa kerangka kaolin CHA sesuai untuk digunakan dalam kejuruteraan tisu tulang terutama untuk aplikasi tanpa galas.

DEVELOPMENT OF CARBONATED HYDROXYAPATITE (CHA) POROUS SCAFFOLDS FOR BONE TISSUE ENGINEERING

ABSTRACT

Carbonated Hydroxyapatite (CHA) powder was synthesized via nanoemulsion method and followed by physico-chemical characterizations. The synthesized CHA powder in this study was confirmed that was B-type CHA in calcium deficient. In the second part of the study, the interconnected porous CHA scaffold was fabrication by polyurethane (PU) sponge replica method. The study was to optimize the composition of CHA slurry by manipulating the type of CHA powders as well as the amount of binder and addition of kaolin to have improved the architecture of the scaffold. Sintering was then performed on the CHA scaffolds at 800℃ with two different heating rate of 5℃/min and 10℃/min for 2 hours. 5wt.% kaolin CHA scaffold with binder ratio of PEG: PVA = 2:3 sintered at 800℃ with heating rate of 5℃/min had the most optimum architecture. The optimum scaffold had 80.11% of apparent porosity with about 200-400 μm of macropores, regular thickness of strut with average of 30-50 μm with minimum crack and compressive strength of 0.072 MPa. It can be concluded that the heating rate used in this study showed the least impact in developing the CHA porous scaffolds whereas the key of success in fabrication CHA porous scaffolds in this study is mainly dependent on the compositions of the slurry used. In terms of bioactivity, kaolin CHA scaffold formed apatite layer on the surface of the scaffold after immersed in SBF for 7 days. This showed that kaolin CHA scaffold was suitable to be use in bone tissue engineering especially for non-bearing applications.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Bone is a natural organic–inorganic composite which comprises about 60% of mineral, 30% of matrix, and 10% of water (Gómez-Morales et al., 2013). Bone is a rigid hard organ in human body and susceptible to fracture due to injury or diseases (Goodrich et al., 2012). One of the treatment to assist in healing is to use synthetic biomaterials as the replacement of the bone lost part (Dorozhkin, 2010). There is a driving force in tissue engineering (TE) field to develop scaffolds that guide bone regeneration across defects that are too large to heal naturally. Bone tissue engineering (BTE) which seeks to apply the scientific principles to engineering of viable substitutes that restore and maintain the function of human bone tissue (Amini et al., 2012; Bose et al., 2012). Bone tissue engineering (TE) scaffolds act as temporary templates for supporting cell attachment and tissue regeneration. Besides, the scaffolds helps to direct the growth of cells seeded within the porous structure of the scaffold or of the cells migrating from surrounding environments (Shuai et al., 2013; Zhou et al., 2010).

Bone scaffolds are typically made of porous degradable materials which can provide the mechanical support during repair and regeneration of damaged or diseased bone (Bose et al., 2012; Lohfeld et al., 2012). Calcium phosphates (CaP) are the main constituents of bone and play as such an essential role in our daily lives. Damaged tissue can best be repaired by something with close resemblance, biomaterials based on CaP were already proposed for fracture treatment in 1920 (Habraken et al., 2015). Calcium phosphate based bioactive ceramic scaffolds which being a major constituent of bone have been extensively studied as scaffold material for bone tissue engineering. They are

called bioactive ceramics, a family that includes mainly hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, tricalcium phosphate **(**TCP), $Ca_3(PO_4)_2$ and biphasic calcium phosphates (BCP, a mixture of HA and TCP) (Champion, 2013; Picard et al., 2018). The current trend of bone substitute material is inclined to regenerative approach where the fast resorption of the implant material and simultaneous filling with newly formed bone and the substituted part, instead of permanently stay at the replaced side (Filippov et al., 2011).

Hydroxyapatite, HA ($Ca_{10}(PO_4)_6OH_2$) is chemically similar to the mineral component of bones and hard tissues in mammals, it is often being used as a substituent of loss part of bone (Bang et al., 2014). The use of granular synthetic HA to repair bone defects has been reported in 1951 (Polo-Corrales et al., 2012). HA ceramics does not exhibit any toxicity effects. It exhibit high bioactivity and biocompatibility with hard tissues and also skin tissues (Suchanek and Yoshimura, 1998). Although synthetic hydroxyapatite (HA) has the ability to form bond with the bone, its solubility and stability is relatively slow (Eliaz and Metoki, 2017). However, it was found that, stoichiometric HA slightly chemically differ from biological apatites where the former is lacking in carbonate ions, which is reported to be the major trace elements besides calcium (Ca) and phosphate (P) (Baba Ismail and Mohd.Noor, 2011). Besides, previously study by Ramesh et al. (2015) reported that HA has poor mechanical reliability, which limit its application clinically in particular for load-bearing orthopaedic and dental applications. Thus, carbonated substituted hydroxyapatite (CHA) ceramic is seen used as an alternative bone material compared to HA-based material. The amount of carbonate ions found in biological bone was reported to be in the range of 2-8 wt. % and it is dependent on the individual's age. Substitution of carbonate ion promotes the solubility or resorption of HA (Ibrahim et al., 2011). Hence, carbonated hydroxyapatite (CHA) is potentially better as its chemical composition approximates bone tissue more accurately.

Generally, there are various synthesis routes have been explored to produce nano-sized HA and CHA based materials, including sol-gel synthesis, hydrothermal synthesis, nanoemulsion method and so on (Bang et al., 2014; Zyman and Mykola, 2011). Nanoemulsion method was commonly use to synthesis carbonated hydroxyapatite due to the simple process, availability of the equipment and the easily adjustable parameters with potentially good results (Germaini et al., 2017).

The powder property may also affect the scaffold property due to the difference in sintering behaviour, dissolution property, etc. However, irrespective of the powder synthesis route, all the scaffold fabrication will start with a powder (Dash, 2015). Therefore, there are different scaffold fabrication route may be adopted which included freeze casting, rapid prototyping, polyurethane (PU) sponge replica method and so on.

1.2 Problem Statement

Polyurethane (PU) sponge replication method has been extensively used in fabrication of CHA porous scaffold with highly interconnected pores. This is because the method involved the coating of PU sponge with CHA slurry with subsequent burn-off of the sponge can result in a porous CHA structure. For all porous structures, their strengths decrease drastically with an increase in porosity and the presented of highly interconnected pores. Therefore, the challenge is to balance the demands of required mechanical strength with architecture which has highly interconnected pore structure (Wheelton， A. et al., 2016). Although the CHA scaffolds prepared by PU sponge replica method possess able to provide highly interconnected pores, they are known to be brittle in nature and poor mechanical strength (Tang et al., 2013).

In practical, sintering is typically performed to densify and enhance the properties of ceramic products. However, sintering CHA porous scaffolds at high temperature (above 800℃) may cause the decomposition of carbonate ions from the CHA structure. It is well accepted that chemically synthesized CHA will start to decompose into HA or calcium oxide with CO_2 being released upon sintering at 800℃ (Bang et al., 2014). The decomposition of carbonate ions will consequently affects the physical and mechanical properties. This is due to poor control of the heat treatment of the CHA (Mohd Noor et al., 2014). The carbonate loss during sintering of CHA must be controlled in order to assure the carbonate amount necessary for adequate biological behaviour of graft is maintained (2-8 wt.%). Therefore, the thermal stability of CHA powder seems to be a key challenge (Landi et al., 2003).

Heat-treatment can be performed in various gas atmospheres, including nitrogen, carbon dioxide, air, water vapour and wet oxygen (Terezinha et al., 2018). It is found that the CHA did not show any evidence of decomposition at temperatures up to 1300℃ when sintered in wet carbon dioxide atmosphere (Barinov et al., 2006; Wong and Mohd Noor, 2016). Besides, the previous study performed by our group found that sintering of CHA with 5 wt.% $Mg(OH)_2$ at 800℃ and by introducing wet carbon dioxide gas at 200℃ during cooling had successfully re-compensate the carbonate loss (Baba Ismail and Mohd.Noor, 2011; Wong and Mohd Noor, 2016). This study had also highlighted the use of $Mg(OH)_2$ as sintering aid, where denser sintered CHA with better mechanical strength and sufficient amount of carbonate retained upon sintering at 800℃ as compared to CHA sintered without $Mg(OH)_2$. To further densify and enhance the mechanical strength of ceramic products, some studies have been investigating on the potential of use of kaolin. This is because kaolin has been well established as a reinforcing agent and shows excellent

mechanical strength and non-toxic properties in commercial biomedical materials (Ballet et al., 2010; Goyanes et al., 2013).

Therefore, the aim of the study is to produce highly interconnected CHA porous scaffold with good physical and mechanical properties. In this current study, CHA porous scaffold will be fabricated via polyurethane (PU) sponge replication method by the addition of kaolin and sintering aid which is magnesium hydroxide, $Mg(OH)_2$. The introduction of kaolin is aimed to enhance the mechanical properties of CHA scaffolds by providing a better framework to the porous scaffolds. While, the additive of Mg(OH)2 as sintering aid is to improve the sintering and it is hoped that higher densification could be achieved at much lower temperature in order to minimize the decomposition of carbonate ions. In this study, wet carbon dioxide gas will also be introduced during cooling stage in order to compensate the carbonate loss during sintering. It is hoped that the fabricated scaffolds will be highly interconnected pores with sufficient strength as well as good chemical and biological properties.

1.3 Research Objectives

The main objectives of this work are:

1. To synthesis CHA powders by nanoemulsion method.
2. To optimise the composition of the CHA slurry in fabricating CHA porous scaffold by polyurethane (PU) sponge replication method.
3. To study the effect of heating rate in fabricating porous CHA scaffolds.

1.4 Scope of Research

This research was divided into four main stages as shown in Figure 1.1.

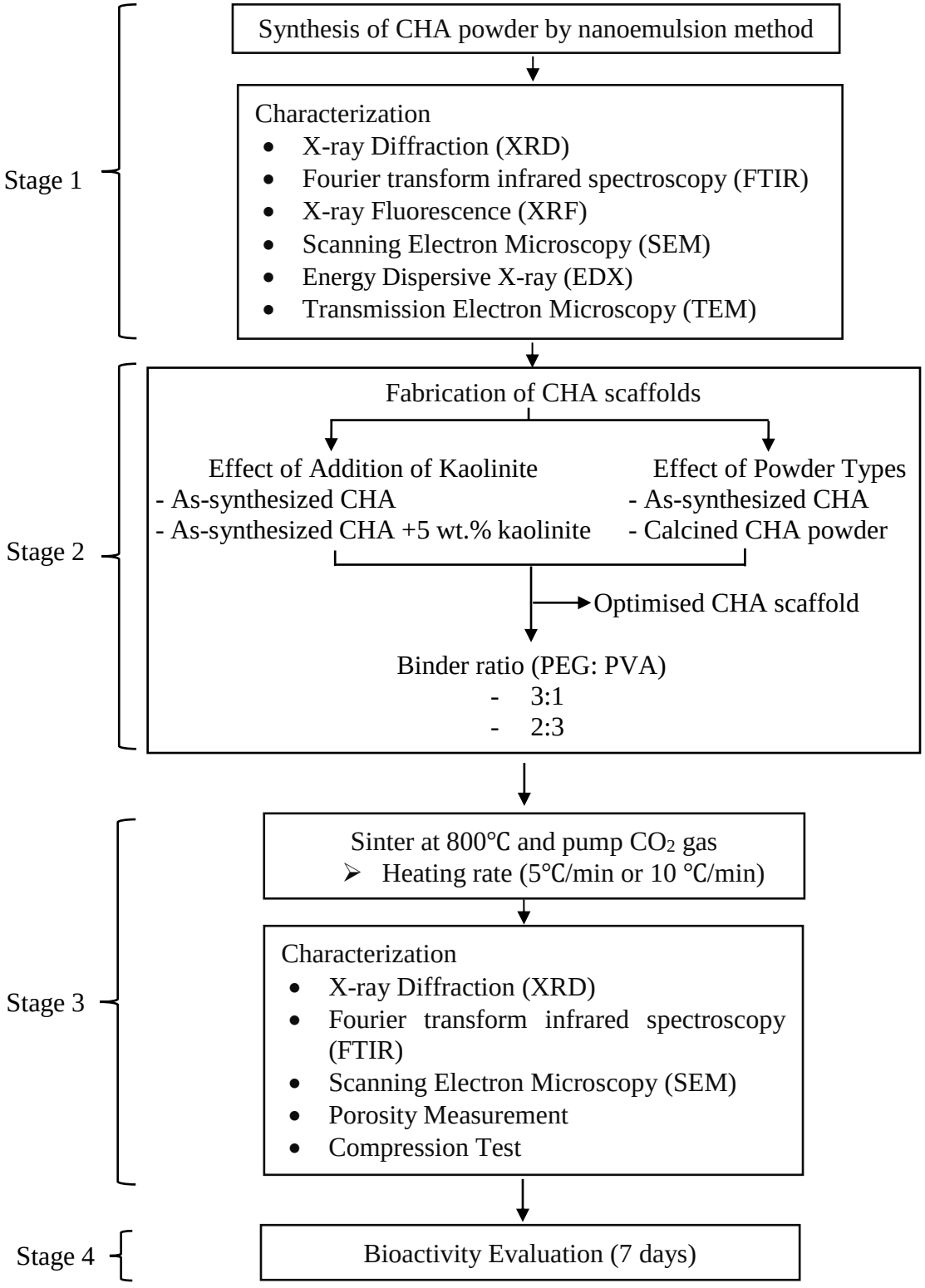

Figure 1.1: Flowchart of overall research work

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This research work study on synthesis of bone scaffold material and fabrication of synthetic bone scaffold as the alternative to bone tissue transplantation for orthopaedic injury treatment. This chapter begins with a brief insight of bone function and composition, hierarchical structure of bone, bone cells and bone remodeling process in order to produce a scaffold which have biomimetic properties with bone. It is followed by bone scaffold for orthopaedics injury treatment. The material selection of bone scaffolds are discussed after understanding the requirement of an ideal bone scaffold. Lastly, the synthesis technique for bone scaffold material and fabrication method of synthetic bone scaffold are presented and discussed.

2.2 Bone Function and Composition

Bones are rigid organs that form part of the endoskeleton of vertebrates. The function of bone is to provide structural support, protect various organs of the body, store cells and mineral ions (Gómez-Morales et al., 2013; Saito, 2011; Sikavitsas et al., 2001). Bone tissue is mostly composed of inorganic mineral salts which termed as biological apatite and organic biological proteins. Bone matrix contains around 65 wt.% calcium phosphate, 25 wt.% collagen as organic materials, and 10 wt.% water (Barrere et al., 2008). Additionally, other organic materials such as proteins, polysaccharides, and lipids are also present in small quantities. Table 2.1 shows the Composition of mineral phase of bone compared with hydroxyapatite (HA).

Table 2.1: Composition of mineral phase of bone compared with hydroxyapatite (HA) (Li, 2010; Saiki et al., 2014; Soares et al., 2007; Teruel et al., 2015; Zenóbio et al., 2011).

Composition	Bone	Hydroxyapatite (HA)
Calcium (Ca) (wt.%)	34.8 - 36.6	39.6
Phosphorus (P)	15.2 - 17.1	18.5
Ca/P (molar ratio)	1.71	1.67
Carbonate (as CO_3^{2-})	4.8 - 7.4	-
Potassium (K)	0.03 - 0.07	-
Magnesium (Mg)	0.60 - 0.72	-
Sodium (Na)	0.9	-
Fluoride (F)	0.03 - 0.10	-
Chloride (Cl)	0.10 - 0.13	-
Total inorganic (wt.%)	65	100
Total inorganic (wt.%)	25	-
Water (wt.%)	10	-

Generally, main, minor and trace elements are used by biologists to distinguish and classified the hard tissues. These elements can cause in very different molecular or crystalline structures (Ferraz et al., 2015; Teruel et al., 2015).The hydroxyapatite (HA) with chemical formula of $Ca_{10}(PO_4)_6(OH)_2$ is the main inorganic mineral phase in biological apatite (Gunderson and Schiavone, 1991; Pasero et al., 2010; Wang et al., 2015). The Ca/ P ratio of HA is less than 1.67. There are some impurities with positively or negatively charged such as CO_3^{2-}, Na^+, Mg^{2+} and so on in natural HA. Besides, there are various of essential trace element exist in biology bone such as silicon (Si), fluorine (F), zinc (Zn), strontium (Sr), magnesium (Mg), boron (B), and copper (Cu), sodium (Na), manganese (Mn), carbonate (CO_3), which play an important role in bone growth (Ferraz et al., 2015; Lin, Chang, et al., 2011; Lin, Zhou, et al., 2011).

2.3 Hierarchical Structure of Bone

Human bones has a fascinating internal and external structure, they are lightweight, yet strong and hard. The hierarchically-structured biomineral of bone with

its particular structure and unique mechanical properties and remodeling capability has been fascinated by scientists (Kumaresan et al., 2015; Umadevi and Geethalakshmi, 2011). Several hierarchical models that range from the nanoscale to the macroscopic scale have been proposed in order to understand its complex architecture and thus the micro-architecture is optimised to bear high loads experienced in everyday situations (Gómez-Morales et al., 2013).

The bone is largely composed of two types of bone tissue which are cortical (compact) bone and trabecular (cancellous or spongy) bone as shown in Figure 2.1 (Athanasiou et al., 2000; Gruber et al., 2008). The ratio of compact bone and the cancellous bone in the adult skeleton are 80% and 20% respectively.

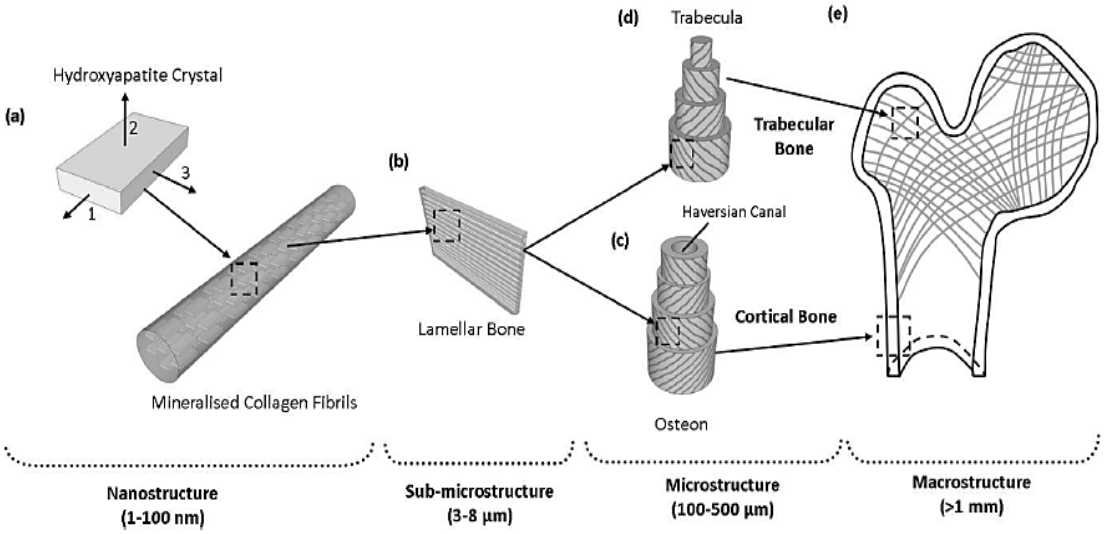

Figure 2.1: Diagram of the hierarchical structure of bone, illustrating (a-b) the composite nature of bone and the bone microstructure organized into (c) osteons and (d) trabeculae. These organizational units respectively form the basic constituents of (e) the cortical and trabecular macrostructure of bone (Vaughan et al., 2012).

Cortical bone is presented as a dense outer shell consisting of highly calcified tissue. Cortical bone is composed of functional units called osteons at the micro-scale. The osteon is also called the Harversian system with an ordered histological pattern. Each

of them contains lamellae that encircle a Harversian canal, a vascular channel about 50 µm in diameter that contains blood vessel capillaries, nerves, and a variety of bone cells as shown in Figure 2.2 (Gray and Standring, 2008; Keaveny et al., 2004). All the canaliculi build a branching network throughout compact bone in order to promote the material exchange between the osteocytes and blood vessels (Sikavitsas et al., 2001; Yi et al., 2016). The collagen fibers are present and oriented parallel in each lamella. Each collagen fiber is composed of different patterns of mineral collagen fibril arrays as shown in Figure 2.3. These patterns can be parallel arrays, woven arrangements, plywood like structures, and radial arrays (Gómez-Morales et al., 2013).

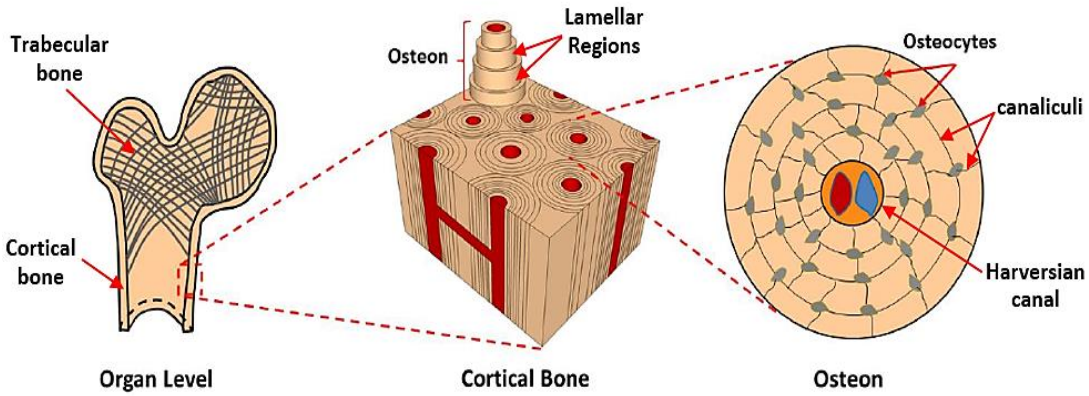

Figure 2.2: Diagram of an osteon, the primary structural unit of bone, with the concentric locations of osteocytes shown (Vaughan et al., 2013).

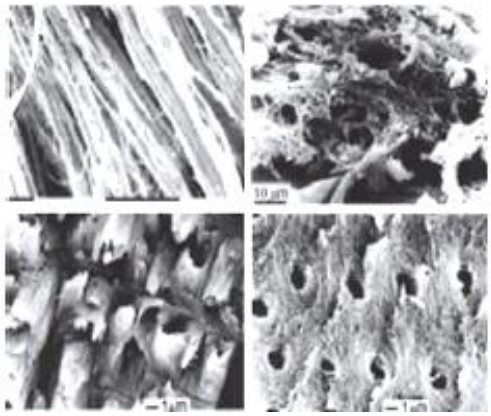

Figure 2.3: Mineral collagen fibril array patterns (Roveri and Iafisco, 2010).

Trabecular bone is found within this outer cortical structure and it has less organized structures as compared to cortical bone. Trabecular bone forms supportive struts which act with the cortical shell to provide optimised mechanical support for minimal bone mass. It has three dimensional highly porous structure with 70 -95% porosity and is filled with bone marrow and bone cells whereas cortical bone is the dense outer layer with about 5–10 % porosity (Keaveny et al., 2004; Verbruggen, 2013).

2.4 Bone Cells and Bone Remodeling

The adult skeleton is continuously renewed by remodeling throughout the lifetime of an individual. Continuous bone resorption and bone formation are essential to provide the ability of bone to adapt to mechanical loads (Kapinas and Delany, 2011; Ruimerman, 2005). Bone modelling is differ from bone remodeling; the former is the process whereby bones are shaped or reshaped by which bone resorption is not coupled to bone formation (Langdahl et al., 2016), while the latter is a dynamic process by which bone resorption is coupled to bone formation, to replace old or damaged bone with new bone (Kapinas and Delany, 2011; McNamara, 2011).

The bone cellular structure consists of three major types of cells constitute bone tissue which are osteoclasts (bone-resorbing cell), osteoblasts (bone-forming cell) and osteocytes (bone-forming cell) (Frost, 2003; Spyros et al., 2010). The properties and functions of these cells are described as below (Dorozhkin, 2011; Mohamed, 2008):

- Osteoblasts: Bone forming cells that responsible for bone matrix synthesis and promote bone formation and mineralization. They act as seeding sites for hydroxyapatite crystal formation through localized enzymatic accumulation of calcium and phosphate.

- Osteoclasts: These cells are large multinucleated cells, like macrophages which act as bone resorbing cells. Osteoclasts mature and migrate to discrete bone surfaces. Active enzymes acid phosphatase are secreted against the mineral substrate upon arrive and result in dissolution.
- Osteocytes: Originated from osteoblasts. Cells lying within the bone itself and become trapped and surrounded by bone matrix. They have many fine canals called canaliculi that allow the diffusion of substances through the bone.

Bone remodeling is mediated by the delicate balance of osteoblast and osteoclast numbers and activities. The old bone is resorbed by osteoclasts and formed new bone by osteoblasts. The process is illustrated in Figure 2.4.

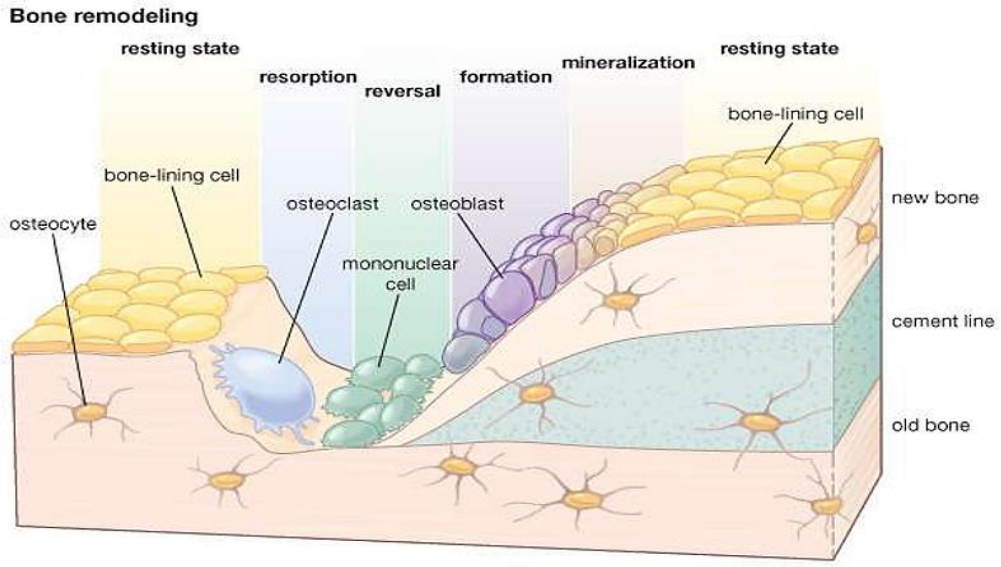

Figure 2.4: Bone Remodeling Process; Remodeling process is accomplished by cycles of resorption of old bone by osteoclasts and the subsequent formation of bone by osteoblasts (Robert et al., 2012).

The bone surface is completely lined by a layer of flattened and elongated cells termed bone-lining cells when bone surfaces are neither in the formative nor resorptive phase. The first step in the bone remodeling cycle is the resorption of existing bone by osteoclasts (Mohamed, 2008). The process is followed by reversal phase which begins

with mononuclear cells preparing the bone surface for new osteoblasts. Next, the cement line is formed in resorption lacunae and osteoblasts. Matrix mineralization and the differentiation of some osteoblasts into osteocytes completes the remodeling cycle (Kapinas and Delany, 2011; Langdahl et al., 2016).

Normally, there is a balance between bone resorption and formation in the adult skeleton and this is a constant dynamic process throughout life (Mohamed, 2008). Therefore, bone remodeling which including the replacement of old and damaged bone with new bone plays a key role in maintaining the mechanical strength of bone (Langdahl et al., 2016). If the balance between bone formation and resorption is lost, the bone structure would be strikingly damaged and susceptible to bone loss and fractures (Klein-Nulend et al., 2012; Ruimerman, 2005).

2.5 Bone Scaffold for Orthopaedic Treatment

Orthopaedic injuries have been a major area of concern in medical field. Disease, injury and trauma can lead to damage and degeneration of tissues in the human body, thus treatments are required to facilitate their repair, replacement or regeneration (Brien, 2011; Oryan et al., 2014). The human bone tissue has high healing ability which capable to heal small defects by itself and without supportive treatments (Kamitakahara et al., 2008). However, its ability to regenerate is limited in the cases of massive defects (Bigham and Oryan, 2015; Bose et al., 2013). Besides, in cases of patients with limited healing, such as those with diabetes or poor nutrition, its regeneration is very slow. So, big defects cannot be spontaneously fixed by the body and thus it requires surgical intervention and the use of a graft (Santos et al., 2012). Figure 2.5 shows radiograph of open tibia fracture with segmental bone loss as a result of trauma injury.

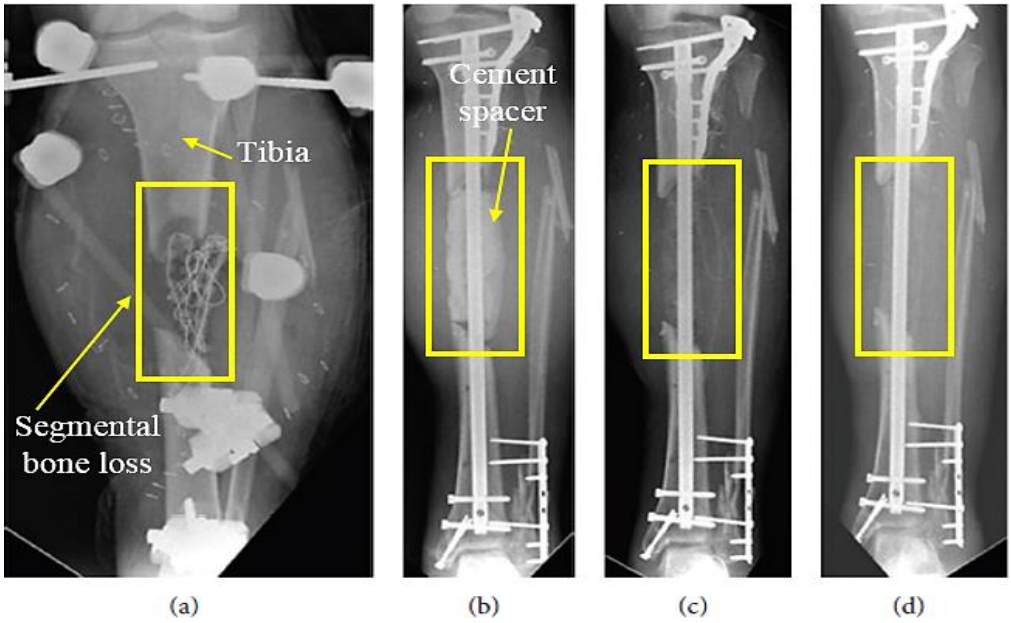

Figure 2.5: (a) Radiograph of open tibia fracture with segmental bone loss as a result of trauma injury. (b) Radiograph of the damaged tibia after internal fixation and filled with cement spacer. (c) Radiograph of the defect after 3 and (d) 4 months. Bone healing never occurred (Pilia et al., 2013).

This is particularly true when large segmental bone defect (SBD) are present, the healing process is extremely prolonged and the bone tissue is difficult to heal on its own as shown in Figure 2.5 (Pilia et al., 2013). Therefore, the bone has failed to return to normal form or function (Karrer et al., 2015).SBD is defined as injuries in which a section of bone is completely shattered and absent, caused by trauma, disease, and age. Normally, the size of the missing section in SBD is large and caused the bone cannot regenerate on its own (Melissa et al., 2016; Pilia et al., 2013).

Bone fractures are known to affect millions of people worldwide and present a serious public health concern. Therefore, the need for tissue regeneration technology grows dramatically along with the world population and the increase in life expectancy (Sturm et al., 2010). Bone graft is second only to blood as the most commonly transplanted tissue (de Azevedo Gonçalves Mota et al., 2016). Current strategies of

treatment consists of autografts (transplant of patients own tissue to sites of injury) and allografts (transplant of tissue from one patient to another) (Wheelton， A. et al., 2016). There still exists some problem in both techniques.

Autografts is considered as the 'gold standard' material for grafting. Its scaffold-like structure allows for cell migration and proliferation (Spyros et al., 2010). The disadvantages of autografts include donor site morbidity, extended operating time, risks of infection and injury of vessels and nerves and autologous graft's limited availability (Baumhauer et al., 2014; Hung and Noi, 2012). Similarly, allograft have serious constraints due to high risks of rejection by the patient's immune system and the possibility of introducing infection or disease from the donor to the patient (Brien, 2011). Although allografts undergo multiple treatments prior to use, risk of disease transmission is still a possibility. The advantages and disadvantages of using autografts and allografts in clinical practice were summarised in Table 2.2.

Table 2.2: Advantages and disadvantages of using autografts and allografts in clinical practice (Roberts et al., 2016; Sheikh et al., 2015).

	Autografts	Allografts
Advantages	• Biocompatible • Osteoinductive • Osteoconductive • Adequate mechanical strength • Available in both cortical and cancellous types	• Availability in large quantities • lack of donor site morbidity • decreased surgical times
Disadvantages	• Need for additional surgery to procure the tissue • Donor site morbidity • Increased risk of fracture to donor site • Increase in operative time and cost • High variability in quality of harvested bone tissue	• Risk of disease transmission and immune reaction • High costs • High-dose irradiation

The concept of tissue engineering emerged in the year 1990 to address the limitations of tissue grafting and repair (de Azevedo Gonçalves Mota et al., 2016; Mahapatra and Khan, 2011; Spyros et al., 2010). Tissue engineering (TE) is an application of biological, chemical, and engineering principles toward the repair, restoration, or regeneration of living tissue by using biomaterials, cells, and growth factors alone or in combination (Amin and Ewais, 2017). Bone tissue engineering attempts to restore and maintain the function of bone tissue using a combination of cells, biological and biochemical signaling molecules and porous 3D scaffolds with no or minimal disadvantages of the bone grafts (Brien, 2011; Schroeder and Mosheiff, 2011). The concept of bone tissue engineering is shown in Figure 2.6.

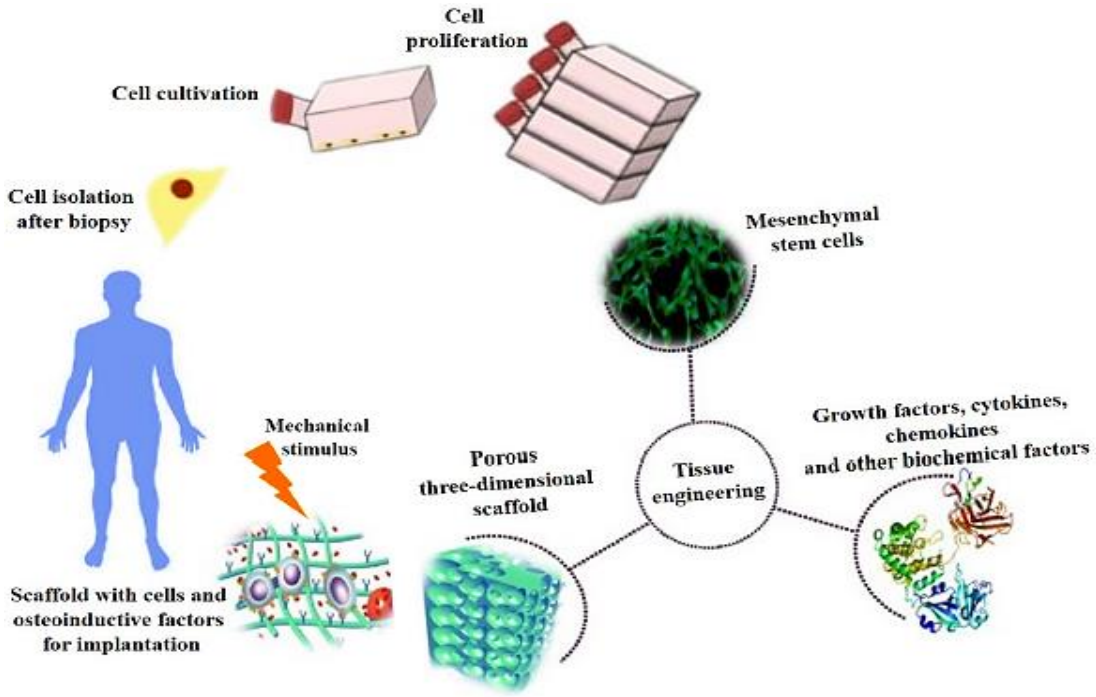

Figure 2.6: Bone tissue engineering. Tissue engineering triad composed of three main constituents including stem cells, growth factors and scaffolds fabricate an engineered tissue to implant into the damaged site (Oryan and Alidadi, 2017).

These scaffolds essentially act as a template for tissue formation and are typically seeded with cells to enable the development of tissue and growth factors (growth stimulating signals) to direct the subsequent interaction between cells and scaffold (Oragui et al., 2011; Thanabalasundaram et al., 2012). These cell-seeded scaffolds are cultured in vitro and then implanted into the bone defects in vivo to induce, accelerate and guide bone formation in the next stages (Chen et al., 2008; Oryan and Alidadi, 2017). This combination of cells, signals and scaffold is often referred to as a tissue engineering triad as illustrated in Figure 2.6.

Successful bone reconstruction intends to facilitate bone healing through osteogenesis (i.e. bone generation) at the site of damage (Gómez-barrena et al., 2015). Bone scaffolds mainly serve as combined functions of mechanical support and bone repair which osteoinductive (i.e. bone inducers) and osteoconductive (i.e. bone guides) capabilities (Gómez-barrena et al., 2015; Wang and Yeung, 2017) . The biological terms of ostegenesis, osteoconduction and osteoinduction can be explained as below:

- **Osteogenesis:** The synthesis of new bone by donor cells derived from either the host or graft donor. Cells involved in this process include mesenchymal stem cells (MMSCs), osteoblasts and osteocytes (Roberts et al., 2016; Roberts and Rosenbaum, 2012).
- **Osteoconduction:** The process by which implanted bone scaffold having a surface that is bioactive and promotes cell attachment and migration, as well allows ingrowth of host capillaries, tissues and cells (Pilia et al., 2013; Roberts and Rosenbaum, 2012).
- **Osteoinduction:** The process by which scaffolds can promote stem cell differentiation through release of local growth factors or hormone. Stem cells are induced to differentiate into osteoblasts and thus facilitate the forming process (Pilia et al., 2013; Wang and Yeung, 2017).

2.5.1 Scaffold Requirement for Bone Tissue Engineering

Tissue engineering (TE) is an interdisciplinary field which intends to combine knowledge of cellular biology, biomaterials and chemical factors to create a functioning scaffold (Cox et al., 2015). A number of key considerations are important when fabricating an ideal scaffold for use in tissue engineering:

(i) Biocompatibility

The key criteria of any scaffold for tissue engineering is that it must be biocompatible which the bone scaffold has ability to prevent an immune reaction or rejection when interacting with the body (Pilia et al., 2013)**.** The scaffold should support cellular activity, conducive to cell adhesion, proliferation and differentiation in order to provide a good micro-environment for cell growth without causing toxic effects to the host tissue (Chandorkar et al., 2015; Tang et al., 2016).

(ii) Biodegradability

Tissue engineering is aimed to allow body's own cells to eventually replace the implanted scaffold construct over time. The scaffold must be biodegradable so that the scaffold degrades as the tissue regenerates and takes its places to allow cells to produce their own extracellular matrix. This is because scaffolds are not intended as permanent implants (de Azevedo Gonçalves Mota et al., 2016; Brien, 2011; Wang et al., 2015) The rate of degradation should be matched with the rate of new bone formation produced by the regenerating tissue to avoid mechanical collapse. Besides, the by-products of this degradation should also be non-toxic. (Pilia et al., 2013; Wheelton， A. et al., 2016).

(iii) Mechanical Performance

The elastic modulus, compressive strength, and toughness are particularly important for the scaffold considered as the load bearing implant. Scaffolds should match the elastic modulus and the ultimate compression strength of natural bone (Dubey et al., 2013; Kumar et al., 2013). Scaffold must have enough mechanical strength to withstand sterilization, surgical handling and transportation during implantation (Brien, 2011). On the other hand, producing scaffolds with adequate mechanical properties is one of the great challenges in attempting to engineer bone. The scaffold would not meet the mechanical strength requirements as its stiffness is too low, it reduces the carrying capacity. Therefore, this situation may cause the bone more prone to fracture (Qin et al., 2014; Wu et al., 2017). Furthermore, the material use to fabricate the scaffolds should be easily machine and able to return to its original shape in the body over time. This implies that biomaterials which have good plasticity are more preferable (Wang et al., 2011).

(iv) Scaffold Architecture

A various of scaffold architectural characteristics including porosity, pore size and permeability play a significant role in biological delivery and tissue regeneration (Li et al., 2013). An ideal scaffold should have highly porous structure with interconnected porosity in order to transporting oxygen, nutrients, and waste metabolites in and out of the scaffold (Igual et al., 2013; Lin et al., 2014). Moreover, large surface area are also required to promote cell adhesion and growth in tissue repair (Macchetta et al., 2009). The ideal pore size and porosity of the scaffold fabricated should match with the normal bone unit. The optimum pore size of the scaffolds is range from 200 μm to 900 μm in diameter in order to encourage cell attachment, migration and ingrowth throughout the scaffold (Murphy and Atala, 2014; Pilia et al., 2013; Tarafder et al., 2011). Wu et al. has been reported that the average size of the human bone unit is about 223 μm.

The ideal porosity of the three-dimensional structure should be ranges from 60 to 99% to allow cell penetration in the regeneration of damaged tissues (Li et al., 2017; Pilia et al., 2013; Wu et al., 2017).

(v) Bioactivity

A bioactive biomaterial is designed to induce a specific biological activity promoting enhanced bone formation and direct chemical bonding of bone to the material surface which known as bonding osteogenesis. There are many biomaterials such as calcium phosphate and other ceramics, polymers and titanium have been exhibited bioactive behaviour (Amin and Ewais, 2017; Overgaard, 2001). The bioactivity of those biomaterials can be evaluated by immersion in the Simulated Body Fluid (SBF). The ability of apatite formation can be evaluated after several days of immersion (Ibrahim et al., 2011; Kokubo and Takadama, 2006). Figure 2.7 shows the example of apatite layer formed on the porous HA scaffold after immersed in SBF.

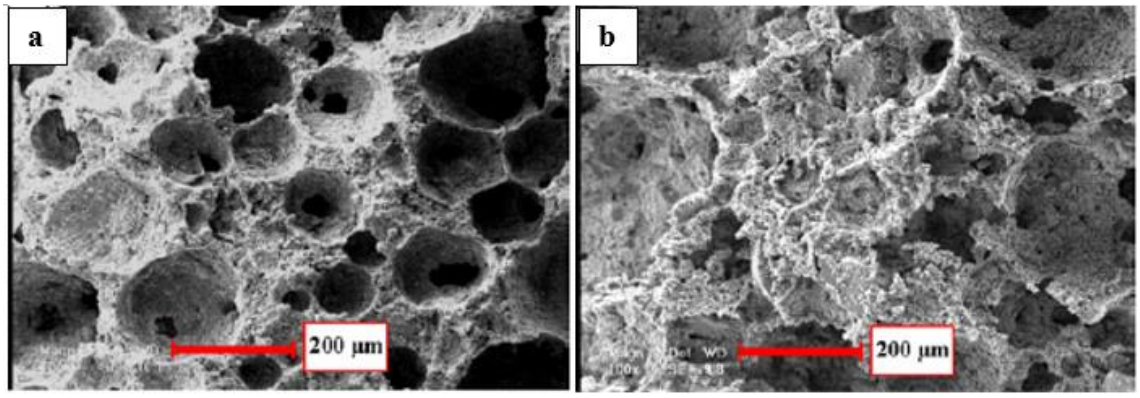

Figure 2.7: Scanning Electron Microscopy (SEM) micrograph of porous HA scaffold shows apatite layer formation: (a) before and (b) after seven days of immersion in SBF (Ghomi et al., 2011).

2.6 Biomaterials

Tissue Engineering (TE) has led to the development of biomaterials to prepare porous 3D scaffolds as biological substitutes to restore, maintain, or improve defective tissues. There are various types of biomaterials including metal, polymers, and ceramics have been proposed for tissue engineering to overcome the problems associated with natural bone grafts in reconstructive surgery (Amin and Ewais, 2017; Brien, 2011).

The biomaterials used in the orthopaedic treatment have been divided into three generations a shown in Figure 2.8 based on the biofunction. The first generation is the development of bioinert material such as stainless steel, titanium, alumina and zirconia. The bionert material does not cause any reaction that interferes with body functions following implantation (Mahyudin et al., 2016; Overgaard, 2001). In the 1980s, the approach changed to another direction so that the goal was to implant the material that can react with the surrounding environment to produce newly formed bone. Therefore, the materials developed in second generation are calcium phosphate and glasses which are bioactive material (Rahaman et al., 2011). Lastly, the third generation of materials are developed by combination of all biological properties such as bioinert, bioactive and biodegrable. The designed material is to stimulate the specific cellular responses at the molecular level, so that the cells able to drive self-regeneration of tissues (Oryan and Alidadi, 2017; Wu et al., 2017). Bioceramics have been developed over the last decades and they have attracted great attention in the field of bone tissue engineering. The most commonly use bioceramic material is calcium phosphate bioceramic which being bioactive, biocompatible, biodegradable and mechanically stiff (Amin and Ewais, 2017).

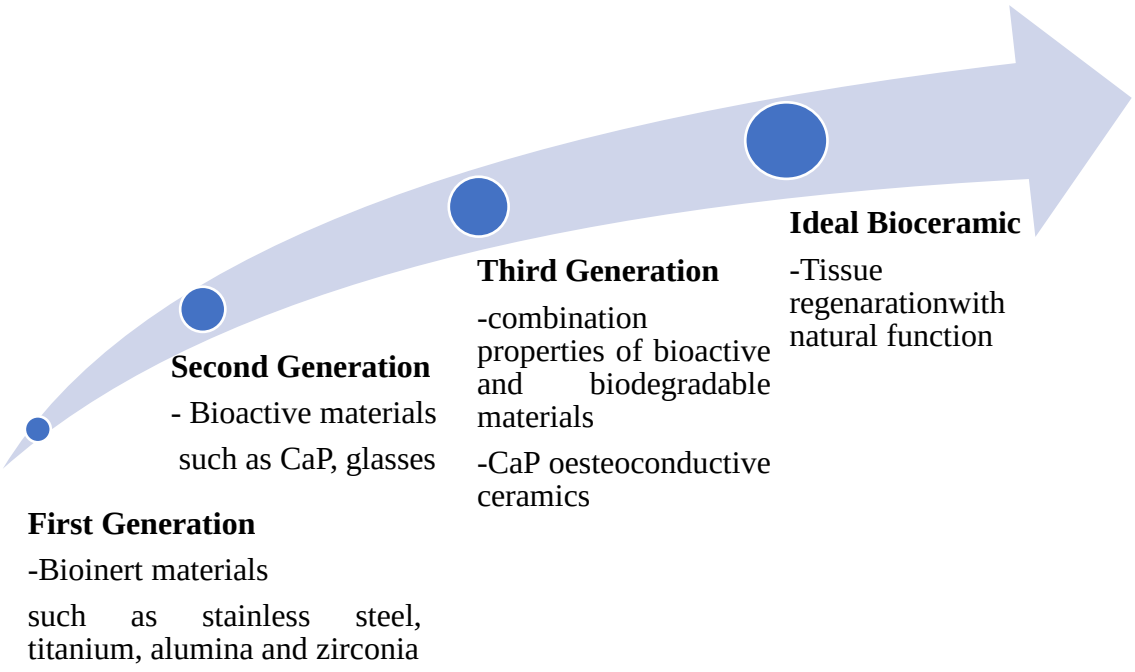

Figure 2.8: Development and evolution of biomaterials (Oryan and Alidadi, 2017)

2.7 Bioceramic

The structural identification of calcium phosphate bioceramic has been established that they have corresponded to an apatite by using X-ray diffraction. Calcium phosphate ceramics are constituted by calcium hydroxyapatites, which is a chemical composition similar to the mineral phase of calcified tissues (Wang and Yeung, 2017). CaP ceramics have received great attention and have been experimented extensively in clinical studies because they are the bioabsorbable ceramic with excellent osteoconductivity (Roberts and Rosenbaum, 2012). Table 2.9 shows the list of the CaP bioceramics with different Ca/P molar ratio. The solubility of the CaP ceramic is mainly based on the Ca/P molar ratio. As Ca/P ratio close to 1.67, their acidity and solubility is decrease significantly while the acidity and solubility of CaP ceramics is extremely high for those have Ca/P ratio less than 1.

Table 2.3: Major calcium phosphates bioceramic (Dorozhkin, 2010; Ferraz et al., 2015)

Ca/P molar ratio	Chemical formula	Name	Abbreviation
0.5	$Ca(H_2PO_4)_2 \cdot H_2O$	Monocalcium phosphate monohydrate	MCPM
0.5	$Ca(H_2PO_4)_2$	Monocalcium phosphate anhydrous	MCPA
1.0	$CaHPO_4 \cdot 2H_2O$	Dicalcium phosphate dihydrate (brushite)	DCPD
1.0	$CaHPO_4$	Dicalcium phosphate anhydrous (monetite)	DCPA
1.2 - 2.2	$Ca_xH_y(PO4)z \cdot nH_2O$ (n = 3 - 4.5)	Amorphous calcium phosphate	ACP
1.5	α-$Ca_3(PO_4)_2$	α-Tricalcium phosphate	α -TCP
1.5	β-$Ca_3(PO_4)_2$	β -Tricalcium phosphate	β -TCP
1.5 - 1.67	$Ca_{10-x}(HPO4)_x(PO_4)_{6-x}(OH)_2$ ($0 < x < 1$)	Calcium-deficient hydroxyapatite	CDHA
1.67	$Ca_{10}(PO_4)_6(OH)_2$	Hydroxyapatite	HA
2.0	$Ca_4(PO_4)_2O$	Tetracalcium phosphate	TTCP

2.7.1 Hydroxyapatite

Stoichiometric hydroxyapatite (HA) Ca_{10} $(PO_4)_6$ $(OH)_2$ has been commonly used in various applications in tissue engineering due to its good biocompatibility and similar chemical composition to the mineral part of bone (Ghomi et al., 2011). Although synthetic hydroxyapatite (HA) has the ability to bond to bone, the rate of osseointegration is relatively slow (Zhu et al., 2012).

Biological apatites are generally crystallizes in the hexagonal $P6_3/m$ space group which is unlike with pure stoichiometric HA that crystallize in monoclinic $P2_1/b$ space group (Combes et al., 2016). Moreover, the main difference between the synthetic hydroxyapatite (HA) and biological apatites is the presence of significant amounts of carbonate ions in all mineralized biological tissues. Therefore, the early studies reported that the potential method for producing synthetic hydroxyapatite is to incorporate ions

that are present in bone mineral, such as carbonate ions, into the HA structure. Early studies on synthetic carbonated hydroxyapatite established that carbonate ions could in fact be part of the apatite structure (Bonfield and Gibson, 2002; Eliaz and Metoki, 2017).

2.7.2 Carbonated Hydroxyapatite

Preparation of a synthetic carbonate substituted hydroxyapatite which known as CHA bone-substitute ceramic that mimics the chemical composition of hard tissue has been presented as a key target of biomaterials research (Bang et al., 2014; Xue et al., 2015). The development of synthetic carbonate-substituted hydroxyapatite has received extensive interest in the past 50–60 years. Substitution of carbonate ions can occur in two distinct atomic sites in the hydroxyapatite lattice. The carbonate group (CO_3^{2-}) can substitute either the hydroxyl (OH^-) or the phosphate (PO_4^{3-}) ions, giving rise to the A-type or B-type carbonation. The amount of A/B ratio in calcium phosphate of human bone mineral is depending on the age. A-type CHA is mostly found in old bone tissue, while B-type is in young bone tissue (Combes et al., 2016; Kee et al., 2013). According to literature, B-type CHA is the preferential carbonate substitution found in the bone of a variety of species due to it is responsible for the decrease of crystallinity and subsequently increases its solubility (Campana et al., 2014; Dorozhkin, 2011; Ibrahim et al., 2011). The A-type and B-type substitution mechanism are shown in chemical equation as in Equation 2.1 and 2.2 respectively (Li, 2010; Stephane et al., 2015):

$$Ca_{10}(PO_4)_6(OH)_2 + xCO_2 \longrightarrow Ca_{10}(PO_4)_6(OH)_{2-2x}(CO_3)_x + xH_2O \qquad \text{Equation 2.1}$$

$$Ca_{10}(PO_4)_6(OH)_2 + xCO_2 \longrightarrow Ca_{10-x/2}(PO_4)_{6-x}(CO_3)_x(OH)_2 + xH_2O \qquad \text{Equation 2.2}$$

Some researches work has been reported that the effect of carbonate ion substitution upon though in vitro and in vivo experiments have confirmed that increases