DEVELOPMENT OF MULTIWALLED CARBON NANOTUBES REINFORCED HYDROXYAPATITE

by

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LIST OF ABREVIATIONS

MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NH_4OH	Ammonia
ACP	Amorphous calcium phosphate
ANN	Artificial neural network
BCP	Biphasic calcium phosphat
BSA	Bovine serum albumin
BET	Brunauer–emmett–teller
Ca	Calcium
$CaCO_3$	Calcium carbonate
$CaCl_2$	Calcium chloride
$Ca(OH)_2$	Calcium hydroxide
$Ca(NO_3)_2$	Calcium nitrate
CaP	Calcium phosphate
CPCs	Calcium phosphate cements
CNTs	Carbon nanotubes
MWCNTs-COOH	Carboxylated multi-walled carbon nanotubes
DAP	Deficient hydroxyapatite
$(NH_4)_2HPO_4$	Diammonium hydrogen phosphate
$Ca(NO_3)_2 \cdot 4H_2O$	Diammonium hydrogen phosphate
$(NH_4)_2HPO_4$	Diammonium phosphate
DCPA	Dicalcium phosphate anhydrate
$K_2HPO_4.3H_2O$	Dipotassium phosphate anhydrate

DMEM	Dulbecco's modified eagle's medium
FFBP	Feed forward back propagation
FBS	Fetal bovine serum
$Ca_{10}(PO_4)_6F$	Fluorapatite
FTIR	Fourier transform infrared spectroscopy
HCl	Hydrochloric acid
HA	Hydroxyapatite
MWCNTs-OH	Hydroxylate multi-walled carbon nanotubes
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
MWCNTs	Multi-walled crbon nanotubes
CCD-18Co	Normal human colon fibroblast cell line
OCP	Octacalcium phosphate
H_3PO_4	Orthophosphoric acid
P	Phosphor
PLlA	Poly l-lactide
PLLA	Poly(l-lactic acid)
PGA	Polyglycolic acid
PLA	Polylactic acid
KCl	Potassium cloride
RBF	Radial basis function
SEM	Scanning electron microscopy
SBF	Simulated body fluid
SWCNTs	Single-walled cnts
NaCl	Sodium cloride
SDS	Sodium dodecyl sulphat

Na_2SO_4	Sodium sulfate
SPS	Spark plasma sintering
TTCP	Tetracalcium phosphate
TEM	Transmission electron microscopy
TCP	Tricalcium phosphate
$(CH_2OH)_3CNH_2$	Tris (hydroxyl-methyl) aminomethane
XRD	X-ray diffractometer
α-TCP	α-tri-Calcium phosphate
β-TCP	β-tri-Calcium phosphate

LIST OF SYMBOLS

cm	Centimetre
°	Degree
°C	Degree Celsius
°C/min	Degree Celsius per minute
g	Gram
h	Hour
L	Litre
<	Less than
m	Meter
ml	Millilitre
mm	Millimetre
min	Minute
>	More than
nm	Nanometer
rpm	Revolutions per minute
%	Percentage
s	Second
T	Temperature
wt %	Weight percent

PEMBANGUNAN HIDROKSIAPATIT YANG DIPERKUATKAN DENGAN TIUB-NANO KARBON

ABSTRAK

Persamaan komposisi kimia hidroksiapatit (HA) dengan fasa mineral tulang dan keserasian biologinya yang sangat baik memenuhi keperluan bahan reka bentuk untuk pembesaran dan pembaikan tulang. Walaubagaimanapun, aplikasi HA dalam peranti menanggung beban adalah terhad disebabkan ciri mekanikal yang lemah. Tiub-nano karbon (CNTs), dengan ciri kekukuhan dan kekuatannya yang baik, mempunyai potensi besar dalam bidang kejuruteraan tisu. Kekukuhan dan kekuatan CNTs, digabungkan dengan saiz yang kecil dan kawasan permukaan antara muka yang besar, mencadangkan CNTs mempunyai potensi yang amat besar untuk dijadikan ejen pengukuhan HA. Dalam kajian ini, komposit HA/MWCNTs/BSA dengan ciri-ciri mekanik ditambah baik untuk digunakan sebagai bahan pengganti tulang telah dibangunkan. Serbuk komposit HA/MWCNTs/BSA dengan pelbagai jenis MWCNTs (MWCNTs-OH, MWCNTs-COOH, MWCNTs) telah berjaya disediakan. Keputusan yang diperolehi menunjukkan bahawa komposit HA yang disediakan dengan menggunakan MWCNTs-COOH dan BSA dengan daya mampatan 29.75 MPa menunjukkan keputusan terbaik berbanding dengan komposit HA yang disediakan dengan menggunakan MWCNTs yang lain. Komposit HA/MWCNTs-COOH/BSA tersebut direndam dalam SBF selama 7 , 14, 21 dan 28 hari pada suhu 37 °C sebagai ujian biologi bertujuan untuk menyiasat kesan SBF terhadap ciri-ciri mekanikal komposit. Daya mampatan komposit telah menurun dari 29.57 kepada 9.11 MPa selepas 28 hari direndam dalam SBF. Nano-HA dengan saiz zarah antara 20 ke 25 nm telah berjaya dihasilkan dengan menggunakan kaedah

pemendakan larutan $Ca(NO_3)_2.4H_2O$ dan $(NH_4)_2HPO_4$. Komposit Nano-HA/MWCNTs-COOH telah berjaya dihasilkan menggunakan kaedah pemendakan. Komposit tersebut dikalsin pada suhu 600, 800 dan 1000 °C. Kekuatan mampatan komposit Nano-HA/MWCNTs-COOH selepas pengkalsinan pada suhu 600, 800 dan 1000 °C adalah masing-masing 27.01 , 27.78 dan 37.43 MPa. Kesan sitotoksik sampel ujian dengan kepekatan yang berbeza telah dinilai dengan assay MTT terhadap fibroblast kolon manusia normal. Pada kepekatan yang rendah, semua komposit didapati tidak sitotoksik apabila dirawat kepada sel-sel fibroblast manusia dan tidak menyebabkan kesan sitotoksik pada percambahan sel, manakala apabila kepekatan sampel ditingkatkan, pengurangan daya maju sel dapat diperhatikan. Komposit HA/MWCNTs-OH/BSA, HA/MWCNTs-COOH/BSA, dan HA/MWCNTs/BSA menunjukkan kesan-kesan percambahan pada sel-sel. Sampel yang digunakan dalam kajian ini tidak menunjukkan kesan toksik apabila percambahan telah didorong. Tambahan pula, Nano-HA/MWCNTs-COOH/BSA membayangkan kesan percambahan pada rangkaian sel CCD-18Co. Komposit HA/MWCNTs-OH/BSA, HA/MWCNTs-COOH/BSA, HA/MWCNTs/BSA dan Nano-HA/MWCNTs-COOH/BSA adalah antara pilihan yang baik untuk membangunkan bahan-bahan ortopedik kerana komposit-komposit ini lulus keseluruhan tapisan, dan kesan yang diingini telah ditunjukkan di dalam badan. Tambahan pula, komposit-komposit ini boleh memasuki pasaran industri dengan kadar kejayaan yang agak tinggi.

DEVELOPMENT OF MULTIWALLED CARBON NANOTUBES REINFORCED HYDROXYAPATITE

ABSTRACT

The similarity of the chemical composition of hydroxyapatite (HA) to the mineral phase of bone and their excellent biocompatibility meets the requirement of materials designed for bone repair and augmentation purposes. However, the application of HA in load bearing devices is limited by its poor mechanical properties. Carbon nanotubes (CNTs), with their outstanding stiffness and strength, have good potential applications in tissue engineering. Their strength and stiffness, combined with their small size and large interfacial surface area, suggest that they may have great potential as a reinforcing agent for HA. In this study, Hydroxyapatite/Multiwalled carbon nanotubes/Bovine serum albomin (HA/MWCNTs/BSA) composites with highly improved mechanical properties for use as bone replacement materials were developed. The HA/MWCNTs/BSA composites powders with different types of MWCNTs (MWCNTs-OH, MWCNTs-COOH, MWCNTs) were successfully prepared. The results show that the HA composites which are prepared by using MWCNTs-COOH and BSA with compressive strength of 29.75 MPa has the highest results in comparison with HA composites which are prepared by using other types of MWCNTs. The HA/MWCNTs-COOH/BSA composite immersed in SBF for 7, 14, 21 and 28 days in 37 °C as in vitro biological test in with the purpose to investigate the SBF effects on mechanical properties. The compressive strength of immersed composites in SBF was decreased from 29.57 to 9.11 MPa after 28 days immersing. The nano-HA with particle size in the range 20 to 25 nm was successfully synthesized using precipitation technique from $Ca(NO_3)_2.4H_2O$ and $(NH_4)_2HPO_4$ solution. The Nano-

HA/MWCNTs-COOH/BSA composites were successfully produced using precipitation technique. The synthesized composites calcined at different temperatures of 600, 800 and 1000 °C. The compressive strength of Nano-HA/MWCNTs-COOH/BSA composites after calcination at 600, 800 and 1000 °C are 27.01, 27.78 and 37.43 MPa respectively. The cytotoxic effect of the test samples with different concentrations was evaluated by MTT assay against normal human colon fibroblast. At low concentration, all developed composites were found to be non-cytotoxic when treated to the human fibroblast cells and did not elicit cytotoxic effects on cell proliferation, whereas when the concentration of samples was increased, the reduction in cell viability was observed. The HA/MWCNTs-OH/BSA, HA/MWCNTs-COOH/BSA, and HA/MWCNTs/BSA composites showed the proliferative effects on the cells. The samples used in the present study did not show toxic effects as proliferation was induced. Furthermore, the Nano-HA/MWCNTs-COOH/BSA implies a cell proliferative effect on CCD-18Co cell line. HA/MWCNTs-OH/BSA, HA/MWCNTs-COOH/BSA, HA/MWCNTs/BSA and Nano-HA/MWCNTs-COOH/BSA composites are possibly a good choice to develop orthopedic materials because these materials passed the entire filter, in which a desirable effect was elicited in the body. Furthermore, these compounds can enter the market industry with a possibly high success rate.

CHAPTER ONE
INTRODUCTION

Nowadays medical world is changing from treating injured organs of bodies by long surgical operations to changing the damaged organ with synthesised implants. Hard tissue and bone substitute materials are mostly synthesised using bioactive and tough materials, which have chemical and structure similarity to the hard tissues. Study of biomaterials includes the use of them in a new composite material with improved properties, modification of the microstructure of the present biomaterials and chemical synthesis of new biomaterials. Material scientists are currently working on projects for the synthesis of biocompatible materials, which mimic the properties of natural bone (Vallet-Regí et al., 2004).

Biomaterials are class of engineering materials, which can be used in animal body, tissue replacement, reconstruction, and regeneration without any long-term adverse effects. Among the different classes of biomaterials, bioceramics is one of the important classes of available material used as human-body implants (Dorozhkin, 2010).

1.1 Bone

Bone is a natural composite material that contains 10 wt.% water, 20 wt.% organic materials, and 70 wt.% minerals (Shi et al., 2006). The organic components mostly comprise collagen fibrils and some cement-like substances, whereas the inorganic mineral materials of bone are composed of calcium-deficient carbonate substituted apatite comprising calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions, which have similar structures to that of hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) (Legeros et al.,

1993). According to its porosity, bone structure is classified into two categories: compact (or cortical) bone and trabecular (or cancellous) bone. Porosity, density, and molecular structure of bone affect its strength and mechanical properties (Rho et al., 1998).

1.2 Bone substitute materials

Ideal bone replacement materials should be biocompatible, nontoxic, osteoinductive and osteoconductive, stable, capable of long-term integration of implant, and possess excellent mechanical properties (Horch et al., 2006; Kao and Scott, 2007). Materials for body repair have been designed and used clinically since 1960s. Many different kinds of synthetic replacements materials have been developed in the last decades as bone replacement materials. Synthetic materials include ceramic-based materials such as calcium phosphate, calcium sulphate, bioglass, polymers, ceramics and composites (Tadic and Epple, 2004; Tadic et al., 2004; Horch et al., 2006; McAllister and Haghighat, 2007; Asti et al., 2008). Composites are combinations of various materials, such as bioactive calcium phosphates and polymers, which can be used as bone replacement materials with improved mechanical properties (Boccaccini and Blaker, 2005, Chesnutt et al., 2009, Xu et al., 2009).

1.3 Hydroxyapatite (HA) and Hydroxyapatite based composite

The composition of HA is similar to the mineral phase of natural bone. HA is an great material for bone replacement, and has been used in clinical applications such as dental and orthopedic applications in bulk form and as filler and coating for more than three decades (Suchanek et al., 1998). However, the poor mechanical

properties of HA have prevented it from being used in major load-bearing devices. The compressive strength of dense HA is four times greater than that of compact bone, but it still has low tensile strength and fracture toughness.

The recent researches in biomaterials is focused on improving the weak points of calcium phosphates and hydroxyapatite ceramics including their low bioresorbability, surface area, bioreactivity, and the biological properties by exploring the special advantages of nanotechnology (Bohner et al., 2012). Nanotechnology has potential to synthesize the inorganic crystals with nano particle size, characterized by a high bioresorbability and surface area, which increase the bioreactivity of crystals. The trend is shifting toward nanotechnology to improve the biological responses of HA, because nano-HA is a constituent of bone (Roveri et al., 2010).

Nano-HA particles are similar to bone crystals, therefore using nano-HA is considered to be very promising for use in in orthopedic application. Nanotechnology offers an exclusive approach to overcome the weaknesses of several conventional materials. Nanotechnology is include the almost all disciplines of human life such as nanomedicine and nanofabrics. Compare to materials with micro size particles, the nano-sized materials are suggest more improved performance, because these materials have higher ratio of surface area to volume and unusual chemical/electronic synergistic effects (Roveri et al., 2010).

Zhou et al. (2011) studied the important role of size and crystal morphology control of nano HA particles for bone tissue engineering. Their review revealed that

developing the HA biomedical materials will benefit from nanotechnology progresses.

Several methods have been used to synthesize HA. The HA was produced through a hydrothermal process by exposing fluorapatite ($Ca_{10}(PO_4)_6F$) to high pressures and temperatures by Levitt at 1996s. Several methods, such as hydrothermal exchange of coral, solid-state reaction between tricalcium phosphate (TCP) and tetracalcium phosphate (TTCP) or between TCP and CaO, as well as wet chemical methods including aqueous precipitation, hydrolysis, and sol–gel techniques, have been used (Akao et al., 1981; Legeros 1993; Weng et al., 1998; Liu, 2001; Bezzi et al., 2003; Vijayalakshmi et al., 2006). Among the different methods of HA synthesis, precipitation method is the most prevalent. It is a simple method because of its ease of operation, tailoring composition, and scaling up for mass production. Precipitation method is a wet chemical reaction between calcium precursors (such as calcium nitrate ($Ca(NO_3)_2$), calcium carbonate ($CaCO_3$), and calcium hydroxide ($Ca(OH)_2$) and phosphate precursors (such as diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) and orthophosphoric acid (H_3PO_4)) under controlled temperature and pH (Kweh et al., 1999; Munguía et al., 2005; Rhee, 2002; Santos et al., 2004).

The properties of HA (including physical, physicochemical, mechanical, and biological properties, as well as heat treatment behavior) and its preparation process are well understood. After decades of research, poor mechanical properties of HA are reportedly its main disadvantage (Hench, 1998; Kweh et al., 1999; Orlovskii et al., 2002). Carbon nanotubes (CNTs) have outstanding mechanical properties and

chemical stability because of their cylindrical graphitic structure. Carbon is one of the known basic foundations in the development of life on earth (Cheng et al., 2009). Since the investigation of CNTs by Iijima in 1991, increasing research has focused on the use of CNTs for reinforcement (Iijima in 1991; Gojny et al., 2003). Their strength and stiffness, combined with their small size and large interfacial area, suggest that they may have great potential as reinforcing agents for HA.

The mechanical properties of CNTs in composites is complicated. The strength and stiffness of CNTs can be transferred to the matrix depending on the amount of interfacial bonding between the two phases. Response of multi-walled CNTs (MWCNTs) to human lung epithelial cells, osteoblast-like cells, and primary osteoblast cells showed that these cells attached and survived on MWCNTs, although minimal proliferation effect is observed. The dimensions and spacing of CNTs may have important role to determining consequent cell spreading and cell proliferation (Giannona et al., 2007).

HA–CNT composites, combining HA and CNTs, can improve mechanical properties of HA for use in a wider range of biomedical applications. The first known publication on HA–MWCNTs composites were introduced in 2004 (Zhao et al, 2004). They combined sodium dodecyl sulphate (SDS)-treated MWCNTs and nitric acid-treated MWCNTs in a solution with $Ca(NO_3)_2$, followed by the addition of $(NH_4)_2HPO_4$ to form HA. The resulting precipitate was dried and hot pressed at 1200 °C for 20 h. Compressive strengths improved from 63 MPa for pure HA to 87 MPa for SDS-functionalized MWCNTs and 102 MPa for nitric acid-functionalized MWCNTs. Xu et al. (2009) reported on the mechanical properties and response of

osteoblast-like cells to HA–MWCNTs composites densified by spark plasma sintering. HA powder and MWCNTs were dry mixed and then consolidated using spark-plasma sintering at temperatures ranging from 900 °C to 1200 °C. Hardness and Young's modulus were evaluated, and osteoblast-like cells were cultured for two and four days. The mechanical properties reportedly increased in hardness and Young's modulus compared with HA. They also reported an increase in cellular response to the composites compared with HA alone (Xu et al., 2009).

Kealley et al. (2006; 2008) reported the only decrease in mechanical properties with the addition of MWCNTs in the literature. They added 2 wt.% MWCNTs with graphite impurities in situ while precipitating HA. The material was then calcined and isostatically hot pressed with 100 MPa at 900 °C in argon. Results showed that the hardness decreased compared with pure HA, whereas the toughness remained the same. The authors attributed the decrease in mechanical properties to the presence of graphite clumps. Another work involving hot pressing was conducted by Meng et al. (2008). HA was made by precipitation method and then calcined at 900 °C. Thin, short MWCNTs were treated with acid and then mixed with HA powder. The authors confirmed that HA remained phase pure during heat treatment, and found 28% improvement in flexural strength and 50% toughness, as evaluated by indentation fracture, both at a loading of 7 vol.%. At loadings higher than this value, mechanical properties decreased (Meng et al., 2008).

Bovine serum albumin (BSA), a protein with similar size of growth factors, was used as a model protein. The BSA release profile and its release efficiency at different BSA adding times were investigated systemically (Yu and Wei, 2011).

Generally, BSA is used as a matrix or soft template to induce calcium carbonate formation (Yao et al., 2009). The improvement in mechanical properties with the addition of BSA can be explained by the fact that proper amounts of BSA can promote CPC crystal growth (Burke et al., 2000; Hovarth et al., 2000; Sadollah et al., 2012).

The addition of BSA can increase the compressive strength of calcium phosphate composites (Chew et al., 2011; Low et al. 2011). The results demonstrated that the addition of BSA and MWCNTs to the CPC caused an increment in the compressive strength of the CPC composite from 1 MPa for pure CPC to more than 14 MPa for CPC/MWCNTs/BSA composite. This further improvement in the mechanical properties might be due to the capability of BSA to promoting CPC crystal growth (Boccaccini et al., 2007).

1.4 Problem statement

With the aging population, the requirement for biomaterials has increased significantly, and more attention has been drawn to hard tissue research. The significant attention on new and biocompatible bone graft materials is ascribed to decades of bone graft usage to repair osseous defects causing from traumas and diseases. More than 800,000 grafting procedures are performed annually (Tusli, 2004).

The development and improvement of synthetic materials for in vivo biological applications is highly desirable as an alternative to allografts and autografts. Hydroxyapatite (HA) has been extensively studied as a hard tissue and bone graft

biomaterial due to its crystallographic and biologically active characteristics stemming from its chemical similarity to the mineral similarity to carbonated apatite in teeth and bones. Synthetic HA is a biologically active calcium phosphate bioceramic that is generally used for coating the orthopedic metal or utilized as bone substitute material (Lange and Donath, 1989; Sadollah et al., 2012). While HA has the ability to promote bone growth along its surface, its mechanical properties, particularly its strength and toughness, are insufficient for major load-bearing applications. Reinforcing HA with a second phase offers a possibility to overcome these limitations.

An ideal reinforcing material would provide mechanical integrity to the composite without negatively impacting its biological properties. Carbon nanotubes (CNTs), considered one of the strongest and stiffest materials available, have excellent potential to accomplish this.. Furthermore, the surface of the CNTs can be functionalised to improve that interaction.

Already, CNTs have shown potential for reinforcing polymers, and to some degree metals and ceramics, as well. Several publications have addressed HA-CNT composites, specifically, but studies thus far have lacked a cohesive view of all aspects of the composites, which encompass production, processing, densification techniques, mechanical behaviour, and biological behaviour. Special attention must be given to the biological impact of the CNTs presence, as current research is conflicting as to whether CNTs might induce a cytotoxic response or perhaps even improve cellular processes (Kealley et al., 2006; White et el., 2007). Regarding the use of CNTs in bone replacement material, another concern is cytotoxicity. There are

several researches that focus on the harmful impact of carbon nanotubes on cell proliferation and adhesive ability. CNTs have excellent mechanical properties and some researches indicated that they are biocompatible materials in human body. (MacDonald et al, 2005; Price et al., 2003; Hu et al., 2004).

Nanoscale hydroxyapatite (nano-HA) is the main component of mineral phase of natural bone. Nano HA powders have greater surface area, thus it can exhibit the improvement in sinterability and enhanced better densification and as a consequence, improve mechanical properties (Legeros, 1993). Nano-HA is supposed to have better bioactivity and biocompatibility and due to its small size and huge specific surface area, they may have exclusive properties. Study by Webster et al. (2000) have revealed that in comparison with micron sized ceramic materials, nano sized materials demonstrated a significant increment in protein adsorption and osteoblast adhesion. A large surface area is necessary for cell attachment and growth. The large pore volume is also required to accommodate and subsequently deliver a cell mass sufficient for tissue repair. The highly porous biomaterials also desirable for the easy deffusion of nutrients to and waste products from the implant and for vascularization, which are important requirements for the regeneration. This research is aimed at synthesis of HA/MWCNTs/BSA, with improved mechanical properties using MWCNTs as reinforcing material.

1.5 Research objectives

The aims of the research is to develop biocompatible composites with highly improved mechanical properties for use as bone replacement materials. The objectives will be achieved through the outlined steps as follows:

i. To investigate the mechanical strength of HA/MWCNTs composites with different types of MWCNTs and BSA.

ii. To synthesis nano-HA/MWCNTs and study the mechanical strength of nano-HA/MWCNTs/BSA composite for use as bone replacement materials.

iii. To investigate the cytotoxicity effect of developed composites via in vitro cell culture.

1.6 Scope of study

This study covers the development of hydroxyapatite composites, characterization of composites and cytotoxicity study. Calcium phosphate-based biomaterials have revealed great performance without side effects on the in vitro or in vivo. However, the final composites have relatively low compressive strength, which limits their applicability in orthopedics. The MWCNTs have been chosen because of their impressive properties, such as mechanical strength, stiffness and it is suitable to apply in biological and biomedical systems. Furthermore, BSA has been chosen because it can act as a promoter of HA crystal growth when bonded to a surface. Thus, the addition of MWCNTs and BSA could lead to the improvement of mechanical properties by modifying the properties of crystallites. The interfacial bonding between HA, MWCNTs, and BSA is an important consideration for composite materials.

HA has the ability to promote bone growth along its surface, but its mechanical properties, especially compressive strength, is not sufficient for load-bearing applications. Nanoscale HA with ultrafine structure and large surface area has been

synthesised for the next step of this research because it has a higher level of biocompatibility compared with conventional HA.

In this study, the biological effects of the developed composites on fibroblast cells are studied and the experimental results are compared with the predicted results of ANN technique. The kinetics of nano-HA precipitation from aqueous solution at the condition of high pH value in range between 10.5 to 11 investigated with purpose of synthesis appropriate nano-HA for use as bone replacement materials. Furthermore, the effect of different temperature on rate of conversion were studied. Synthesized composites are characterized through surface area analysis, scanning electron microscopy (SEM), surface and pore size measurements (Brunauer–Emmett–Teller, BET), transmission electron microscopy (TEM), X-ray diffractometer (XRD) and Fourier transform infrared spectroscopy (FTIR).

1.7 Organization of the thesis

This thesis is included five major chapters. Each chapter represents an integral part of the main work and is sequentially arranged. The thesis is organized as follows.

Chapter one provides an overview of the overall research project. The problem statement is written after reviewing different types of bone replacement materials. The research objectives, scope of study, and organization of the thesis are provided in this chapter.

Chapter two presents a detailed review of literature on the properties, characteristics, and production of calcium phosphate-based composites. It also reviews relevant studies on MWCNTs, BSA, and the current status of MWCNTs as reinforcement materials. The chapter also reviews an ANN method that can be used as a potent tool to predict the cytotoxic properties of composites. Finally, a literature review on the kinetic study of the precipitation reaction of HA synthesis is presented.

Chapter three focuses on the materials used and the experimental processes which employed to synthesis of composites. This chapter also presents in detail the experimental methodologies used in the synthesis of different composites and the synthesis of nanoscale HA. It briefly describes the characterization of the composites.

Chapter four presents and discusses the results obtained in this work. The first section of this chapter contains results and discussion of the production of calcium phosphate based composite, in vitro study of composites, and characterization of the synthesized composite. The succeeding part includes the formation of HA/MWCNTs/BSA using conventional HA and different types of MWCNTs, followed by characterization of the composites. The third part of this chapter contains a discussion of nano-HA synthesis through precipitation reaction and preparation of HA/MWCNTs/BSA composite using nano-HA, followed by characterization of composites. In last part, the cytotoxicity of the composites was assessed with human fibroblasts cells (CCD-18 Co), which were inoculated and exposed to different concentrations of different types of sample powder.

Chapter five presents a summary of the results of this research and offers recommendations for future studies related to this research work.

CHAPTER TWO
LITERATURE REVIEW

The purpose of this chapter is to give a background relating to bone replacement materials (particularly CPC and HA composites) and CNTs. Detailed information relating to processing techniques and characterization; Heat treatment; mechanical properties and biological responses of the composite materials; kinetic study and mathematical analysis will be included in chapter 3 and 4. This literature give an overview of the bone nature and different types of materials currently use or under development that can replace bone and assist to repair it. The background on CNTs, which chosen as reinforcing material for CPC and HA is also elaborated. Furthermore, mechanical properties and cytotoxicity of composites are discussed.

2.1 Bone

Bone is an organ with critical functions in human physiology. Some of these functions include protection, movement and support of other organs, blood production, mineral storage and homeostasis, and blood pH regulation (Porter et al., 2009). Bone is a complex natural organic–inorganic ceramic that consists of collagen fibrils embedded with well-arrayed nano crystalline rod shaped inorganic material 25 nm to 50 nm in length (Poinern et al., 2009; Zhou and Lee, 2011). Bone stores 99% of the total body calcium, whereas the remaining 1% circulates in the blood (Rho et al., 1998).

2.1.1 Bone Structure

Figure 2.1 shows that the structural order in bone occurs at different hierarchical levels and reflects the materials and mechanical properties of its components. It also shows that the microstructure of compact bone comprises of osteons with haversian canals and lamellae; at the nanoscale, the structural units are collagen fibers comprised of bundles of mineralized collagen fibrils (Zhou and Lee, 2011). Hydroxyapatite (HA) with a general formula of $Ca_{10}(OH)_2(PO_4)_6$ is the main inorganic material component of bone (Habraken et al., 2007; Hutmacher et al., 2007).

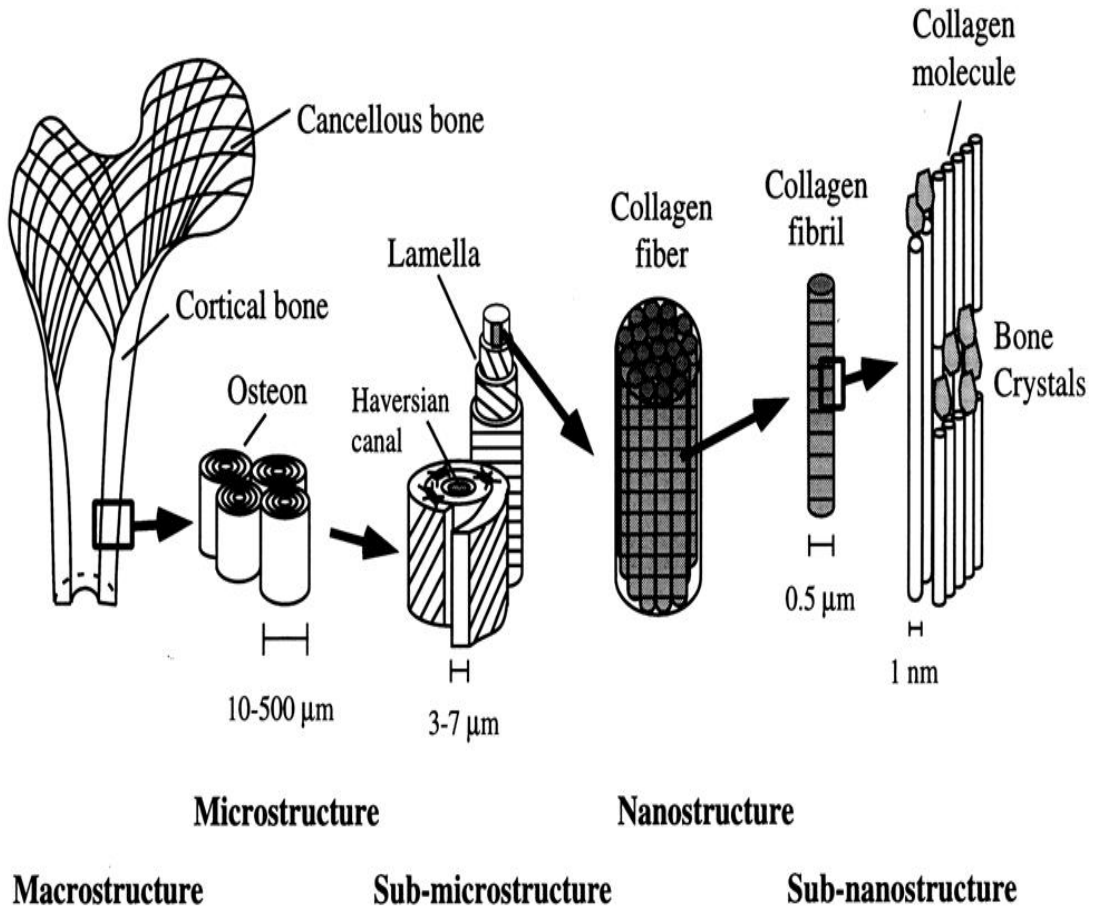

Figure 2.1: The hierarchical structure of bone (Zhou and Lee, 2011)

Despite the diversity of external forms of bone, its internal structure is relatively consistent. Bone structure is hierarchically organized (Weiner and Wagner, 1998; Hellmich and Ulm, 2002). Skeleton comprised from two hundred six bones with different size, shape and weight. This complex internal and external structure is responsible for 9 kg of adult human weight. Bones are rigid, living tissue, constantly tearing down and rebuilding them, without this repair and reinforcement of even minor weak spots, we would break bones on a regular basis. Bone serves multiple functions, including mechanical, synthetic, and metabolic. Through mechanical function, bone provides the frame work for the body; also provide protection for internal soft tissue like brain, heart, liver and muscle. Bone and several associated elements cartilage, connective tissue, vascular elements, and nervous components act as fundamental organ generating movement force for the body (Gilbert, 2001).

Bone is multifunctional tissue and not only having protective function. The spongy tissue inside interior cavity of long bones and interstices of cancellous bone produces immature cells, called stem cells, which later develop to red blood cell. Bone marrow produces other elements of the blood such as white blood cells and platelets (Haywood, 2008).

In addition to mechanical function, bone serve metabolic role by reserving minerals, fats, and acid-base balance. The bone stores 99% of the body's calcium and 85% of the phosphorus. The yellow bone marrow of long bones acts as storage of fats. Bone balances acid, and base in blood, when acid production is increased, renal net acid excretion, and kidney function is not enough to buffer the condition, bone

release of Ca^{2+} into the urine, balances the condition (Frost and Saunders-Smith, 2001; Lemann et al., 2003).

Bone structure is not uniformly solid material; it consists of both living tissue and non-living substances. Generally, bone is categorized into two types: Cortical bone, also known as compact or dense bone and Trabecular bone, also known as cancellous or spongy bone, this classification is done base on porosity and the unit microstructure. Cortical tissue give bone smooth, white, and solid appearance, its porosity is 5-30%. Compact bone is account for 80% of the total bone mass of an adult skeleton. Twenty percent of remaining of total bone mass is called trabecular (cancellous or spongy). It is the porous or cellular structure, which fills the interior of short bones and flat bones as well as in the inner part of bony tuberosities under muscle attachments. Its porosity reaches to 30–90% (Wolstenholme et al., 1956; Currey, 2002; Symposium, 2009).

Bone cell classified to three groups of cell osteoclast, osteocblast, and osteocytes. Osteoclast cell are bone marrow originated cell line, they are leucocytes related cell line. Osteocblast, is the differentiated osteogenic cells and is responsible for the synthesis and mineralization of new bone therefore they called as bone-forming cells (Green, 1994). Osteocytes are the inner cell and the most abundant cell type in the bone, comprising more than 90% of all cells within the bone matrix or on bone surfaces the inner cell of the bone. They derived from osteoblasts during the formation of new bone. Osteocytes create network between bone cells, endothelial cells, and hematopoietic cells (Link, 2013). Osteoclast dissolute mineralized matrix of bone, and release the minerals, this process called bone resorption breaking up the

organic bone, and end in release calcium in blood (Figure 2.2) (Green, 1994; Symposium, 2009).

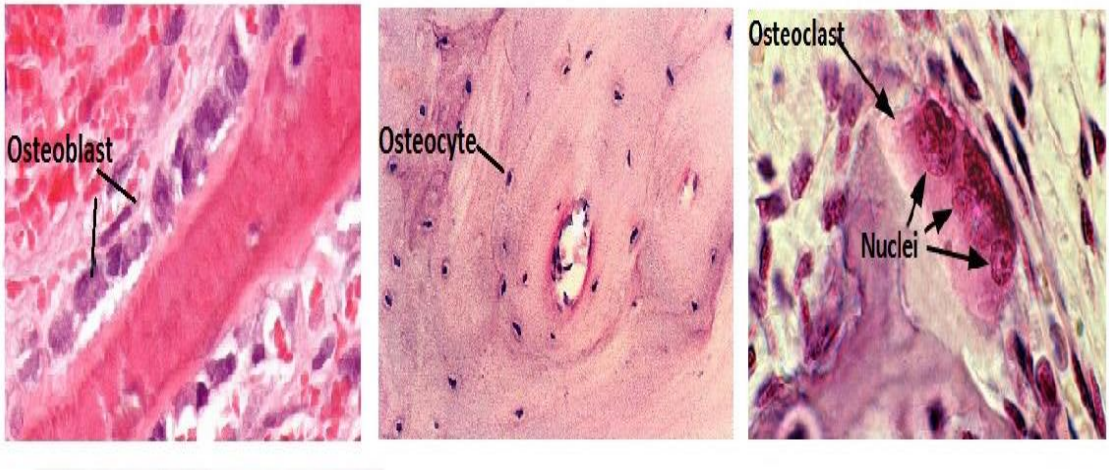

Figure 2.2: Bone cells classified to three groups Osteoblast, Osteocyte, and Osteoclast (Zhang, 1999).

Bone at the lowest level made of remarkably complex arrangements of fibrous protein, collagen (organic composition), strengthened with hydroxyapatite crystals (inorganic composition), there some other material like water, unknown protein, polysaccharide. Water is very important as it determined the mechanical behavior and provide medium for organic and inorganic compound which together make bone matrix (Field et al., 1974; Legros et al., 1987).

At the macrostructural level of hierarchical organization (observation scale of several millimeters to centimeters), bone structure is classified into cortical (or compact) bone and trabecular (or cancellous) bone according to its porosity. Cortical bone porosity is in the range of 5% to 10% and usually found along the exterior shaft section of long bones. Cortical bone forms the outer shell around the trabecular bone in joints and in vertebrae (Jacobs, 2000). Trabecular bone with porosity ranging from

75% to 95% is usually found in cubicoidal and flat bones (e.g., vertebrae and pelvis) and in the end of long bones (e.g., femur). Trabecular bone accounts for approximately 80% of the skeletal volume and 70% of the skeletal mass is cortical bone. The microstructure of trabecular bone is composed of irregular, sinuous convolutions of lamellae, and the microstructure of cortical bone is comprised of regular, cylindrically shaped lamellae. Cortical bone is composed of osteons or Haversian canals. The osteons are embedded in a matrix of lamellar bone known as interstitial lamellae (Rho et al., 1998).

Lamellae are bands or layers of bone (3 μm to 7 μm thick) forming an anisotropic matrix of mineral crystals and collagen fibers (Jacobs, 2000). Trabecular packets and osteons are composed of lamellae and are attached to the bone matrix by cement lines. At the molecular level (in the scale of ≤100 nm), three main important materials exist, namely, biologic apatite crystals, collagens, and noncollagenous organic proteins. Mature crystals are not needle shaped but plate shaped (Rho et al., 1998; Weiner and Wagner, 1998). Plate-like biological apatite crystals of bone occur within the isolated spaces within the collagen fibrils. This phenomenon limits the possible primary growth of the mineral crystals and forces the crystals to be discrete and discontinuous (Rho et al., 1998; Weiner and Wagner, 1998). The nanocrystalline bone apatite has small but significant amounts of impurities, such as HPO_4, Na, Mg, citrate, carbonate, and K. Similar to the crystal of HA, the crystal of carbonate apatite not only exhibits a hexagonal crystal structure but also produces X-ray diffraction patterns (Weiner and Wagner, 1998). Weiner et al. (1999) proposed that their plate-like structure is attributed to their growth by an octacalcium phosphate transition

phase. Octacalcium phosphate crystals are plate shaped and have a similar structure to apatite, except for the presence of a hydrated layer.

2.1.2 Mechanical Properties of Bone

The mechanical properties of trabecular and cortical bones including compressive strength, tensile strength, Young's modulus, hardness, and fracture toughness have been extensively studied. Differences between whole bones perhaps explained by the variation in the structure and mechanical function of bones (Rho et al., 1998). The mechanical properties of cortical bone (tibia, femur, and humerus) are significantly influenced by the porosity, mineralization level, and organization of the solid matrix. The mechanical properties of cortical bone vary between subjects, although the density remains the same. In contrast to cortical bone, in trabecular bone, the mechanical properties of the humerus, proximal tibia, and lumbar spine are not different (Rho et al., 1995; Zhou and Lee, 2011).

The strength and mechanical properties of bone depend on bone density, porosity, and molecular structure. Other factors, such as, humidity, mode of applied load, direction of the applied load, and location of bone within the body, affect the mechanical properties of bone (Rho et al., 1998).

2.1.3 Bone and implantation

Bones are relatively rigid structures and their shapes are closely related to their functions. Bone metabolism is mainly controlled by the endocrine, immune, and neurovascular systems, and its metabolism and response to internal and external stimulations are still under assessment (Ratner et al., 2012). Disturbance to normal

function and metabolism of bone causes with any stimuli, ended in bone disease, and need of different treatment. Long bones are most part of skeletal system to injury, and internal or external fixation is a part of their treatment. Joint replacement is main intervention where the bone is expected to host biomaterials. Response of the bone to biomaterial arbitrates with the regeneration procedure. Materials implanted into the bone, nevertheless, cause local and systemic biological responses even if they are known to be inert (Ratner et al., 2012).

Biomaterials are the natural or synthetic material that is used to replace or restore function of body tissue. Currently the wide ranges of materials used in medicine and biotechnology. These materials are mainly divided into four classes; polymers, metals, ceramics, and natural materials (including those from both plants and animals). Different classed of this material can be mixed to develop composite material with increased biocompatibility, efficacy in treatment; end result will be calss fifth which call composite material. Examples of the fifth class of biomaterial are silica-reinforced silicone rubber or carbon fiber- or hydroxyapatite particle-reinforced (Ratner et al., 2012).

The application of exogenous material in treatment was started back in 1960s, during World War II. Surgeons had used commercially available polymers and metals, fabricating implants and components of medical devices from them, as replacement for lost part in the body (Thomas, 2004). The first generations of use biomaterial in implantation happen to be impressively effective; however there were a few failure rejections due to infection. This achievement in medication made surgeons to consider the physical, biological, and materials science and engineering,

where by the earliest interdisciplinary “bioengineering” collaborations were born. These collaborative research teams not only focus to control the biomaterial composition, quality, and purity, they also established the need for new materials with new and special properties. This stimulated the development of many new materials in the 1970s (Thomas, 2004; Ratner et al., 2012).

Biomaterials were specifically designed to be physically replaced the hard or soft tissues, which suffer from a variety of destructive processes including fracture, infection, and cancer that cause pain, disfigurement, or loss of function. Suture is one of the oldest and literary the first generation of biomaterial used in wound healing process. Back to 2000 b.c, the ancient Egyptians used linen to holding together the edges of a wound or surgical incision. Later by developed technology synthetic suture material like polymer (the most widely used) and some metal stainless steels and tantalum were replace with it. The used biomaterial not summarized to wound healing device also a wide range of biomaterials with multiplicity of properties are used to produce ocular devices to correct functional deficiencies caused by disease, age and ocular trauma (Lloyd et al., 2001).

Metals, ceramics and polymers used to replace defected part in the cardiovascular, or circulatory system. The heart valves suffer from structural changes that prevent the valve from either fully opening or fully closing and arteries, particularly the coronary arteries and the vessels of the lower limbs, become blocked by fatty deposits (atherosclerosis), all these can be successfully treated with implants. Bacterial infections, dental caries (cavities), the demineralization and dissolution of teeth associated with the metabolic activity in plaque, cause extensive damage to the

tooth and supporting gum, raise the need of tissue replacement. The need of tissue replacement introduces another area for use of biomaterial. Therefore, verities of material use to replace segment or entire teeth in their entirety.

One of the fastest growing areas for implant applications is devices for controlled and targeted delivery of drugs. This area of implantation focus on the synthesis, fabrication, and evaluation of biomaterials, including nanobiomaterials for important applications in biomedicine. Drug delivery implantation emphasize the development of novel biomaterials with exciting or improved physical, chemical, and biological properties to control drug delivery and selectivity, and increased the efficacy of drug.

Orthopedics implant is the most dominant area for application biomaterials; free movable joint in skeleton like hip, knee, shoulder, ankle, and elbow, affected by chronic condition of osteoarthritis and rheumatoid arthritis. These chronic conditions developed by genetic and environmental factors, aging joints, previous injuries, and obesity affect the structure of synovial joints and induce considerable pain in joints especially weight bearing joint and disturb the joint movement . The interruption in ambulatory function quite devastating but it has been possible to replace these joints with orthopedic implants products. Wide range of metals, polymers, ceramics, and composites used within the manufacture of orthopedics implants the relief of pain and restoration of mobility. There are many deal to be made before the material can be produced and these summarized in Figure 2.3.

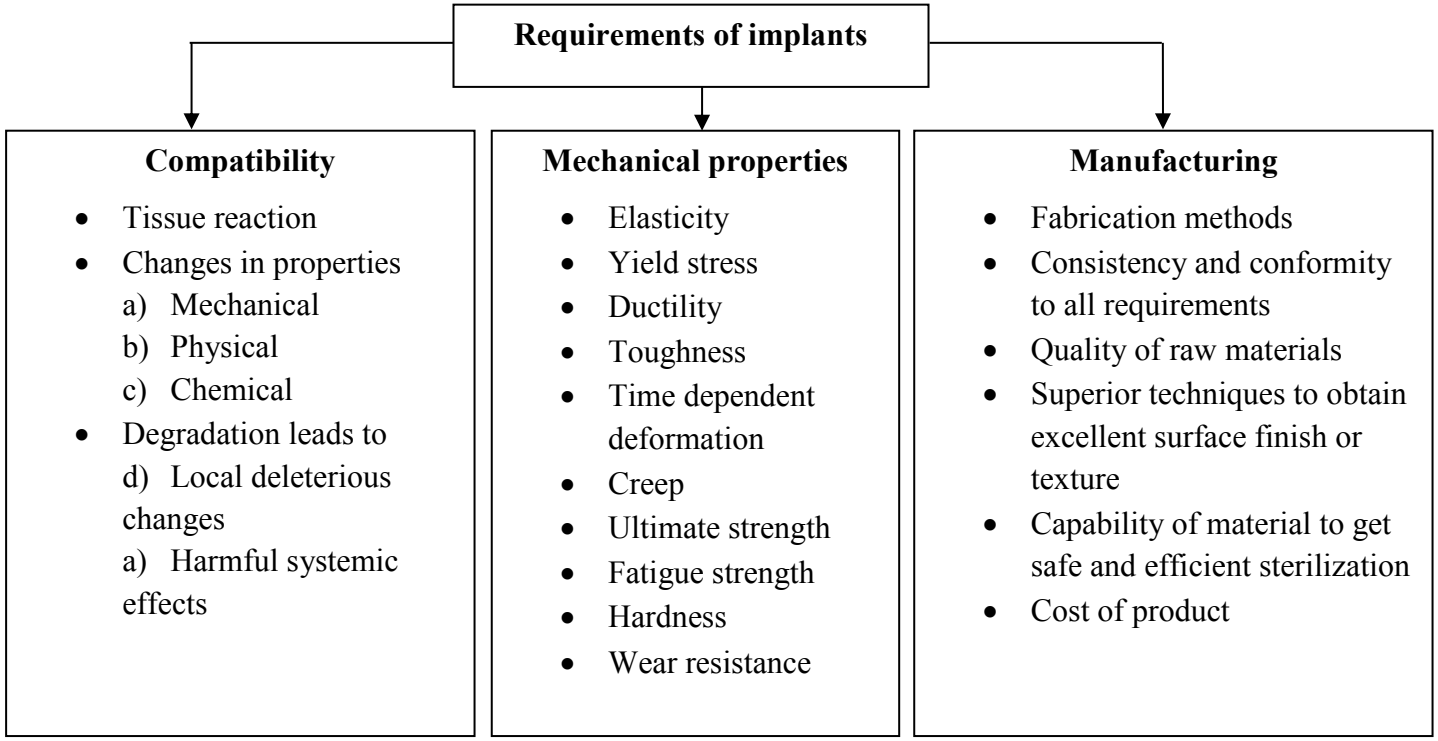

Figure 2.3: Implant material requirement in orthopedic application