SYNTHESIS AND CHARACTERISATION OF CALCIUM PHOSPHATE NANOSHELLS USING DOPA AND DPPA LIPOSOME TEMPLATES

YEO CHIEW HWEE

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SYNTHESIS AND CHARACTERISATION OF CALCIUM PHOSPHATE NANOSHELLS USING DOPA AND DPPA LIPOSOME TEMPLATES

By

YEO CHIEW HWEE

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LIST OF ABBREVIATIONS

ACP	Amorphous calcium phosphate
Ag	Silver
Al_2O_3	Alumina
Au	Gold
α-TCP	α-tricalcium phosphate
BCP	Biphasic calcium phosphates
BSA	Bovine serum albumin
β-TCP	β -tricalcium phosphate
C	Carbon
C-H	Carbon-hydrogen
C=O	Carbonyl
Ca	Calcium
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride anhydrous
CaOH	Calcium hydroxide
$CaNO_3$	Calcium nitrate
CaP	Calcium phosphate
Ca/P	Calcium-to-phosphorus
CDA	Calcium deficient apatite
CDHA	Calcium-deficient hydroxyapatite
CEPA	2-carboxyethylphosphonic acid
CHA	Carbonate hydroxyapatite
Cl	Chloride
CNTs	Carbon nanotubes
Co	Cobalt
CO_2	Carbon dioxide
CO_3	Carbonate
CO_3^{2-}	Carbonate ion
COOH	Carboxyl
CPCs	Calcium phosphate cements
DCP	Dicalcium hydrogen phosphate
DCPA	Dicalcium phosphate anhydrous
DCPD	Dicalcium phosphate dehydrate
DI	De-ionised
DLS	Dynamic light scattering
DMPA	1,2-dimyristoyl-*sn*-glycero-3-phosphate (sodium salt)
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphotidylcholine
DOPA	1, 2-dioleoyl-*sn*-glycero-3-phosphate (sodium salt)
DPPA	1, 2-dipalmitoyl-*sn*-glycero-3-phosphate (sodium salt)
EDX	Energy-dispersive X-ray spectrometer
FDA	Food and Drug Administration
FeO	Iron Oxide
FESEM	Field emission scanning electron microscope
FTIR	Fourier transform infrared spectroscopy
GUV	Giant unilamellar vesicles
HA	Hydroxyapatite
H-O-H	Water
H_3PO_4	Phosphoric acid

HPO_4^{2-}	Hydrogen phosphate
ICDD	International center of diffraction data
KBr	Potassium bromide
LUV	Large unilamellar vesicles
LDV	Laser Doppler velocimetry
L/P	Liquid-to-powder ratio
MCPM	Monocalcium phosphate monohydrate
MLV	Multilamellar vesicles
MRI	Magnetic resonance imaging
MVV	Multivesicular vesicles
MWCNTs	Multi-walled carbon nanotubes
MWCNTs–COOH	Carboxylated multi-walled carbon nanotubes
MWCNTs–OH	Hydroxylated multi-walled carbon nanotubes
N_2	Nitrogen
Na	Sodium
Na^+	Sodium ion
NaOH	Sodium hydroxide
$NaNH_4HPO_4•4H_2O$	Ammonium phosphate tetrahydrate
NH_3	Ammonia
NH_4	Ammonium
NO_3	Nitrate
O or O_2	Oxygen
OCP	Octacalcium phosphate
OH^-	Hydroxide ion
O–H	Hydroxyl
OLV	Oligolamellar vesicles
o/w	Oil-in-water
P	Phosphorus
PA	Phosphatidic acid
PE	Phosphatidylethanolamine
PLA	Poly(lactide acid)
PLGA	Poly(D,L-lactic-co-glycolic acid)
PO_4^{3-}	Phosphate ion
Si	Silicon
SiO_2	Silica
SUV	Small unilamellar vesicles
T_c	Transition temperature
TCP	Tricalcium phosphate
TEM	Transmission electron microscopy
TTCP	Tetracalcium phosphate
w/o	Water-in-oil
wt %	Weight percentage
XRD	X-ray diffraction
ZrO_2	Zirconia

LIST OF SYMBOLS

α	Alfa
β	Beta
θ	Radiation angle for X-ray diffraction analysis

SINTESIS DAN PENCIRIAN NANO-KELOMPANG KALSIUM FOSFAT MENGGUNAKAN TEMPLAT LIPOSOM DOPA DAN DPPA

ABSTRAK

Kalsium fosfat (CaP) adalah biobahan yang sangat berguna dalam kejuruteraan tisu tulang disebabkan ciri-ciri biologi mereka yang istimewa. Kesukaran utama yang dihadapi dalam kebanyakan kaedah-kaedah ialah pengawalan saiz dan bentuk zarah-zarah CaP. Oleh itu, tujuan projek ini adalah untuk mensintesiskan nano-kelompang CaP dengan menggunakan liposom sebagai templat supaya saiz dan bentuk zarah-zarah CaP boleh dikawal. Keberkesanan liposom (*1, 2 dioleoyl-sn-glycero-3-phopshate* (garam natrium) (DOPA) dan *1, 2-dipalmitoyl-sn-glycero-3-phosphate* (garam natrium) (DPPA)) sebagai templat disiasat dalam permulaan projek ini. Keputusan menunjukkan kedua-dua liposom membentuk struktur-struktur bulat liposom *unilamellar* di mana memenuhi keperluan morfologi dan saiz templat nano-kelompang CaP dengan menggunakan kaedah penyediaan yang sesuai. Kesan kepekatan NaOH (0.025 M, 0.050 M, 0.075 M, 0.100 M atau 0.125 M) dan ion-ion kalsium (Ca^{2+}) (0.050 M, 0.100 M, 0.150 M atau 0.200 M) bagi ciri-ciri nano-kelompang CaP disediakan dengan menggunakan templat liposom DOPA dan DPPA dikaji seterusnya. Natrium hidroksida (NaOH) memainkan peranan sebagai satu penyelesaian asas untuk meningkatkan pH dan mengawal penepuan lampau campuran nano-kelompang CaP. Ca^{2+} pula ialah satu lagi parameter utama yang mempengaruhi elektrostatik setempat dengan templat liposom dan pertumbuhan CaP pada templat liposom. Morfologi, nisbah molar akhir kalsium-kepada-fosforus (Ca/P), saiz zarah, taburan saiz zarah, keupayaan zeta, kumpulan berfungsi dan fasa nano-kelompang CaP telah dinilaikan dengan

mikroskop elektron imbasan pancaran medan (FESEM), mikroskop elektron penghantaran (TEM), spektrometer sinar-X serakan tenaga (EDX), *Nano Zetasizer ZS*, spektroskop inframerah jelmaan Fourier (FTIR) dan pembelauan sinar-X (XRD). Daripada penyelidikan dengan kepekatan NaOH dan Ca^{2+} yang berbeza, hasilan terbaik dalam projek ini menunjukkan nano-kelompang CaP yang bulat dengan nisbah molar akhir Ca/P 0.97 terbentuk pada pH 10.52 dengan menggunakan templat DOPA apabila 0.100 M NaOH dipadankan dengan 0.100 M Ca^{2+} digunakan. Di sebaliknya, zarah-zarah berbentuk jarum atau tidak teratur telah diperhatikan dalam nano-kelompang CaP disediakan dengan templat liposom DPPA. Penyediaan nano-kelompang CaP dengan menggunakan templat liposom DOPA dan DPPA telah ditunjukkan sebagai habluran sebahagiannya (yang digalakkan dalam aplikasi perubatan) atau amorfus dalam analisis FTIR dan XRD. Keputusan menunjukkan CaP yang disediakan tanpa templat mempunyai zarah-zarah terkumpul yang besar, tidak stabil dan dihablurkan disebabkan pertumbuhan penukleusan seragam di mana menceburi mekanisma pertumbuhan penukleusan-pengagregatan-pengaglomeratan. Sebagai tambahan, dari mekanisma pertumbuhan, ia telah didapati bahawa kestabilan templat liposom boleh mempengaruhi pertumbuhan penukleusan heterogen nano-kelompang CaP di mana adalah satu faktor mustahak dalam sintesis nano-kelompang CaP.

SYNTHESIS AND CHARACTERISATION OF CALCIUM PHOSPHATE NANOSHELLS USING DOPA AND DPPA LIPOSOME TEMPLATES

ABSTRACT

Calcium phosphates (CaPs) are very useful biomaterials in bone tissue engineering due to their excellent biological properties. The major difficulties facing in most of the synthesising methods are the size and the shape-controlling of CaP particles. Therefore, the aim of this project is to synthesise CaP nanoshells by using liposomes as template in order to control the particle size and the shape of the CaP particles. The effectiveness of liposomes (1, 2 dioleoyl-*sn*-glycero-3-phosphate (sodium salt) (DOPA) and 1, 2-dipalmitoyl-*sn*-glycero-3-phosphate (sodium salt) (DPPA)) as template were firstly investigated in this project. The results showed that both liposomes formed spherical structures of unilamellar liposomes, in which fulfil the morphology and the size requirement of templates of CaP nanoshells by using suitable preparation method. The effect of concentrations of sodium hydroxide (NaOH) (0.025 M, 0.050 M, 0.075 M, 0.100 M or 0.125 M) and calcium ions (Ca^{2+}) (0.050 M, 0.100 M, 0.150 M or 0.200 M) to the properties of CaP nanoshells prepared using DOPA and DPPA liposome templates were then studied. NaOH plays the role as a base solution to increase the pH, and thus, control the supersaturation of the mixture of CaP nanoshells. In addition, Ca^{2+} is another main parameter to influence the electrostatic localisation with liposome template and the growth of the CaPs on the liposome template. The morphology, final calcium-to-phosphorus (Ca/P) molar ratio, particle size, particle size distribution, zeta potential, functional group and phase of CaP nanoshells were evaluated by field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM),

energy dispersive X-ray spectrometer (EDX), Zetasizer nano ZS, Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD). From the investigation of different concentrations of NaOH and Ca^{2+}, the best results in this project showed that spherical CaP nanoshells with Ca/P molar ratio of 0.97 were formed at pH of 10.52 by using DOPA liposome template when 0.100 M of NaOH coupled with 0.100 M Ca^{2+} were used. In contrast, the needle or irregular shaped particles were observed in the CaP nanoshells prepared with DPPA liposome template. CaP nanoshells prepared using DOPA and DPPA liposome templates were indicated as poorly crystalline (favorable in biomedical application) or amorphous in FTIR and XRD analyses. Results showed that the CaPs prepared without template had large and unstable of agglomerated and crystallised particles due to the homogenous nucleation growth, in which involved growth mechanism of nucleation-aggregation-agglomeration. In addition, from growth mechanism, it was found that the stability of liposome template can influence the heterogeneous nucleation growth of CaP nanoshells, in which is an important factor in the synthesis of CaP nanoshells.

CHAPTER ONE:

INTRODUCTION

This chapter provides the detail of introduction for this project. Brief definitions and advantages of calcium phosphate (CaP) nanoshells are included in the beginning of the chapter. The problem statement, scope of study, objectives and thesis organisation of this project are also included in this chapter.

1.1 Calcium Phosphates (CaPs) as Bone Biomaterials

CaPs including hydroxyapatite (HA), alumina (Al_2O_3), zirconia (ZrO_2), silica (SiO_2) based glasses or bioactive glasses (Shi, 2006), generally termed as biomaterials which have both biochemical compatibility and biomechanical compatibility (Cao and Hench, 1996). Over the last decades, research in the field of CaPs has been increased very rapid. The number of published journal articles related to CaPs has rapidly increased as shown in Figure 1.1. This shows the importance of this field of research. These biomaterials have been used as bone biomaterials such as bone substitutes or bone replacement (Giannoudis *et al.,* 2005; Paul and Sharma, 2006; Mastrogiacomo *et al.,* 2006; Habibovic and Barralet, 2011; Fergal, 2011). They have also been used to guide and develop the bone healing tissue, to become integrated within it and then subjected to the same remodelling process as the natural bone (Frayssinet *et al.,* 1998). Moreover, they can help in achieving the best possible level of care for patient's sake.

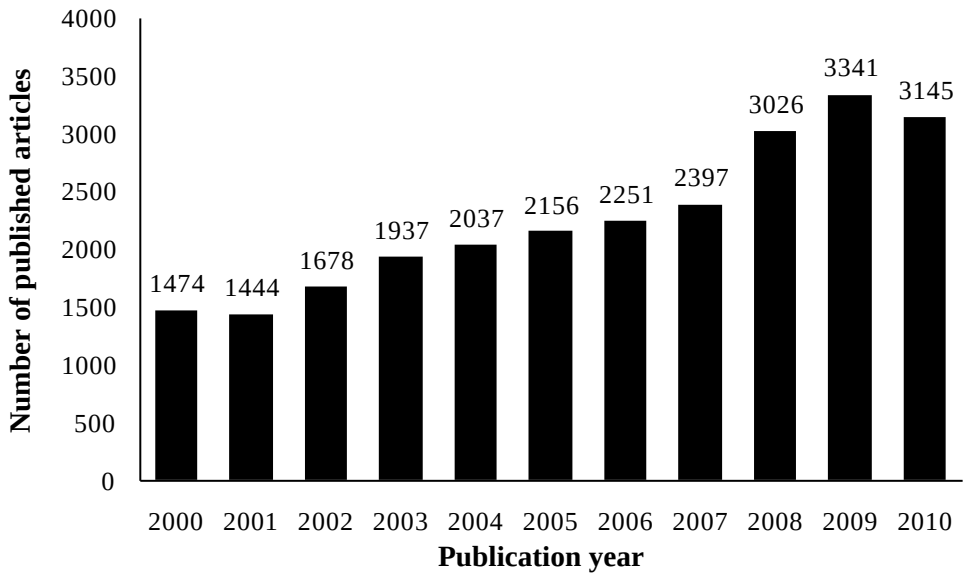

Figure 1.1: Number of published articles related to CaPs as a function of publication year (Data obtained at 29 March 2011 through the ISI web of knowledge search).

CaPs are composed of ions commonly found in the physiological environment (e.g., calcium (Ca), sodium (Na)) (Hulbert *et al.*, 1972), which make them highly biocompatible. In addition, these biomaterials are also non-toxic, non-allergic, and also resistant to microbial attack, pH conditions (de Groot *et al.*, 1990; Hench, 1998; Kalita *et al.*, 2007). Thus, CaP biomaterials are very useful biomaterials. However, the applications of CaP biomaterials have been limited to small, unloaded and lightly-loaded implant (Shi, 2006) due to their very poor mechanical properties.

1.2 Why Calcium Phosphate (CaP) Nanoshells?

Nanotechnology has achieved the position as one of the critical research endeavors of the early 21st century (McNeil, 2005). The study of CaPs as biomaterials is a specific area in nanotechnology. An obvious advantage of nanotechnology as it relates to biological systems is the ability to control the size of the resulting particles (Parashar *et al.*, 2008). Many advances have been made in

biomaterials with the rapid growth of nanotechnology. According to many reports, the size of apatite in biological hard tissues always possesses a range of a few to hundreds of nanometers (Weiner and Addadi, 1997; Weiner and Wagner, 1998; Boskey, 2003).

CaPs have been used widely in medicine and dentistry application as bone biomaterials due to their excellent biological properties. Although CaPs can fulfil some of the characteristics of bone, CaP still have some limitations in clinical applications such as poor adaptation to the shape of bone cavities and fixation problems when granules are used (Fernández *et al.* 1999a; Fernández *et al.* 1999b). This is due to the potential of CaPs used *in vivo* depend upon their ability to withstand complex stresses at the site of application and their compatibility with the biological environment (Kalita *et al.,* 2007). Besides, CaPs have very poor sinterability and poor mechanical properties such as very low compressive strength. This limits the applications of CaPs to small, unloaded and lightly-loaded applications such as osteoconductive coatings on metallic prosthesis and as nano-powders in spinal fusion. Moreover, surgeons reported on difficulties in filling the vertebral bodies (a bad injectability of present formulations) (Dorozhkin, 2008). The poor injectability also reduces the application of CaP biomaterials.

In addition, the mechanical and biological performance of CaPs depends highly on the chemical composition and physical characteristics such as structure, crystal and particle size (Rey, 1990; Best, 1994; Lu *et al.,* 2002a). Thus, it is important in controlling these characteristic features in order to improve the performance of CaPs. Nanotechnology is one of the approaches, which has been

explored recently to improve both the strength and toughness of CaPs to make them useful in load-bearing applications (Kalita *et al.,* 2007). Many of the deficiencies of CaPs such as poor mechanical strength, poor sinterability and poor injectability can be improved if the CaPs prepared in nanoscale structure (Bohner and Baroud, 2005; Kalita *et al.,* 2007). For example, nanostructured HA demonstrated excellent chemical and microstructural uniformity and mechanical properties, compared to conventional HA (Ahn *et al.,* 2001). In addition, it was verified that nanocomposites in nature show a standard mechanical structure in which the size in nanoscale of mineral particles are used to ensure optimum strength and maximum tolerance of flaws (Gao *et al.,* 2003).

However, the research in the synthesis of CaP nanoshells is yet to be extensively studied although the number of published articles for the formation of nanoshells has increased rapidly in the past decades as shown in Figure 1.2. CaP nanoshells are hollow solution-filled nanoparticles in size range of 20 nm to 200 nm that prepared by coating liposomes with nanometre thick layer of inorganic CaPs. The small sizes and spherical shape of nanoshells make them ideal for injection to the human body (Ishikawa, 2003; Wingert *et al.,* 2007; Dorozhkin, 2008; Sounderya and Zhang 2008). Thus, CaP nanoshells are promising candidates for used in medical and dentistry application. Moreover, various synthesis routes have been developed over the past few years to prepare nanoshell based materials such as interfacial polymerisation (Scher *et al.,* 1998; Vincent, 2006), layer-by-layer deposition (Ai *et al.,* 2003; Vincent, 2006), sol-gel method (Pal and Chakravorty, 2005; Chatterjee, *et al.,* 2005; Basu and Chakravorty, 2006) and others. Thus,

synthesis of CaP nanoshells is the most important issue to be addressed as it controls the properties of the final CaP nanoshells.

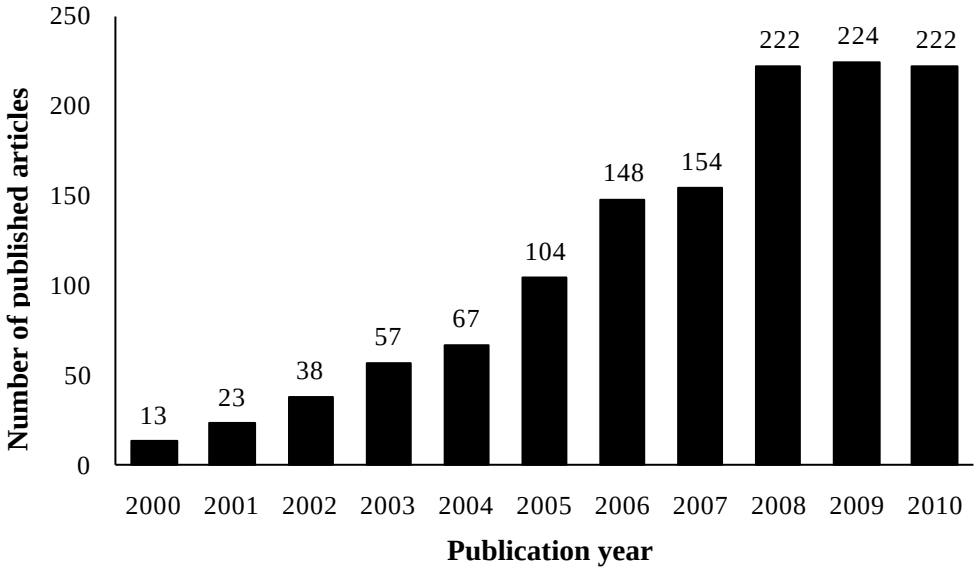

Figure 1.2: Number of published articles related to nanoshells as a function of publication year (Data obtained at 29 March 2011 through the ISI web of knowledge search).

1.3 Problem Statement

The major problem created by bone disease, especially osteoporosis, is fractures, which may be the first visible sign of disease in patients (Office of the Surgeon General, 2004). Osteoporosis affects an estimated 75 million people in Europe, USA and Japan (European Foundation for Osteoporosis and The National Osteoporosis Foundation, 1997). In Malaysia, it is estimated that over 1 million people are at risk of osteoporosis, out of which 80 % are women (Arthritis Foundation Malaysia, 2011). CaPs are the biomaterials of choice in both dentistry and medicine in order to solve the problem. The recent trend in this field of research is focused on overcoming the limitations of CaPs and in improving their biological properties by exploring the unique advantages of nanotechnology (Kalita *et al.*, 2007). In addition, the great challenge in this research field is to develop synthesis

method that is able to control the particle size and shape of CaPs uniformly (Hu *et al.,* 2010).

In this research, CaP nanoshells by using 1, 2-dioleoyl-s*n*-glycero-3-phosphate (sodium salt) (DOPA) and 1, 2-dipalmitoyl-*sn*-glycero-3-phosphate (sodium salt) (DPPA) liposomes as template will be synthesised in order to control the particle size and shape. The small sizes and spherical shape of nanoshells make them ideal to be used in the human body such as ease for injection to the body (Ishikawa, 2003; Dorozhkin, 2008; Sounderya and Zhang 2008). Since the shape of the materials can be controlled by the shape of the template used (Pileni, 1998), the synthesis method using a template are being explored. For example, Tjandra *et al.* (2006) reported the use of polymeric template to synthesise hollow spherical CaP nanoparticles. However, the removal of this polymeric template is needed. Thus, liposomes are preferred in this research because they are non-toxic, biodegradable and non-immunogenic in nature, and thus, they can remain in the human body (Lasic, 1995). In addition, the negatively charged polar headgroup (-OH) of liposomes can assist in the localisation of calcium ions (Ca^{2+}) around the liposomes (Schmidt *et al.* 2004). Nevertheless, the effectiveness of template in controlling the shape of CaP nanoshells can be affected since some discrepancies arise in the synthesis using template (Pileni, 2003). Therefore, the various parameters affecting their size and shape still need to be investigated in order to produce CaP nanoshells which have better properties compared to existing CaP materials. In this project, sodium hydroxide (NaOH) plays the role to increase the pH, and thus, to control the supersaturation of the mixture of CaP nanoshells (Schmidt, 2006). Moreover, the addition of NaOH is known to have a large impact on the particle formation

(Nishimura *et al.,* 2011). In addition, Ca^{2+} is another main parameter to influence the electrostatic localisation with liposome template and the growth of the CaPs on the liposome template (Schmidt *et al.,* 2004). Hence, in this research, NaOH and Ca^{2+} are the main parameters to be investigated in the synthesis of CaP nanoshells using DOPA and DPPA liposome templates.

1.4 Research Objectives

The main goal of this study is to synthesise the CaP nanoshells by using liposomes as template in order to control their particle size and shape. The objectives in this project are as following:

i. To investigate the effectiveness of DOPA and DPPA liposome templates
ii. To synthesise CaP nanoshells by using DOPA and DPPA liposome templates
iii. To study the effect of concentrations of NaOH and Ca^{2+} to the properties of the synthesised CaP nanoshells using DOPA and DPPA liposome templates
iv. To study the growth mechanism of the synthesised CaP nanoshells

1.5 Scope of Study

The first step in this project is to study the DOPA and DPPA liposomes. The effectiveness of both liposomes to be used as template in the synthesis of CaP nanoshells is investigated. The results are crucial as basic information required to prior to conduct the further experimental works.

Secondly, CaP nanoshells by using DOPA and DPPA liposomes templates are synthesised. The effect of the experimental conditions for concentrations of NaOH and Ca^{2+} on properties of CaP nanoshells is also studied. Moreover, CaP nanoshells with and without liposome templates are studied and compared in the research work.

Transmission electron microscopy (TEM), field emission scanning electron microscope (FESEM), energy-dispersive X-ray spectrometer (EDX), Zetasizer nano ZS, Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) are used to characterise the physical and elemental properties of the CaP nanoshells. TEM is used to investigate morphological of liposomes and CaP nanoshells in very high resolution. The main purpose of using FESEM and EDX are to study surface topography of CaP nanoshells and to determine the composition of elements presented in the final products, respectively. The particle size and zeta potential of the liposome templates and CaP nanoshells are measured by using Zetasizer nano ZS. FTIR is used to observe the development of functional groups and XRD is used to determine the phases formed.

Lastly, growths mechanisms of the formation of CaP nanoshells with and without liposome templates are developed based on the results of characterisation. The growths mechanisms are useful to understand the synthesis of CaP nanoshells.

1.6 Organisation of The Thesis

This thesis consists of five chapters. Chapter one provided an outline of the overall research project including the introduction of CaPs as biomaterials and the

reason the CaPs need to be prepared in nanoshells. Project statement was written after reviewing the existing CaPs. This revealed the problems faced and the importance of this research project. The objectives of this research project were then carefully formulated with the intention to address the problems. The main objective here was to synthesise the CaP nanoshells by using liposomes as template in order to control the particle size and shape of CaP nanoshells. Besides, the effect of concentrations of NaOH and Ca^{2+} to the properties of the CaP nanoshells and also their growth mechanism were studied.

Chapter two contains the background information of various research works reported in the literature in this area of study which includes types of CaP biomaterials, type of liposomes and their preparation methods, various methods to synthesise CaP nanoshells. In addition, the potentials applications of CaP nanoshells are discussed.

The experimental materials and methodology used in this research are discussed in chapter three. This chapter describes the details information on the overall flow of this research works and the experimental methods in conducting the project. The synthesis parameters to be investigated and the characterisation techniques of the CaP nanoshells are also described here.

Chapter four is the heart of this thesis as it includes the detail discussion on the results obtained in the research work. This chapter is divided into four sections according to the stages and experimental conditions of this research work. First section presents the characterisation of prepared liposomes that had been done

before further experimental works are carried out. Section two reports the effect of concentrations of NaOH and Ca^{2+} the properties of CaP nanoshells by using DOPA and DPPA liposome templates. Section three represents the comparison between CaP nanoshells with and without liposome templates produced under optimum conditions. At the end of this chapter, growth mechanism of CaP nanoshells with and without liposome templates also discussed.

Chapter five, the last chapter of the thesis, gives a summary on the results obtained in this project. This chapter also gives some recommendations for future studies related to this project.

CHAPTER TWO:

LITERATURE REVIEW

This chapter reported the literature related to this research project which is CaP nanoshells prepared by using liposomes as template. Overview of CaP based bone biomaterials, types of CaP biomaterials, nanoshells, CaP nanoshells, types of liposomes and their preparation method are provided. Various synthesis methods of liposome based CaP nanoshells and their potential applications are explained in more detail.

2.1 Brief Introduction on Calcium Phosphate (CaP) and Nanoshells

CaPs have attracted great attention in medicine and dentistry as bone biomaterials due to their excellent biocompatibility and bone-repair properties. In fact, the mineral fraction of hard tissues is composed of sparingly soluble CaPs (Fernández *et al.*, 1999c). The requirements of bone biomaterials especially bone substitutes are good local and systemic compatibility, the capability of being substituted by bone and of entirely filling any flaw. These features require osteoconductive and/or osteoinductive properties of the bone substitutes, thus CaPs are the primary materials of choice for their application.

Although CaPs can fulfill the characteristics of bone, CaPs still have some limitations in clinical applications such as poor adaptation to the shape of bone cavities and fixation problems when granules are used (Fernández *et al.*, 1999c). Moreover, the poor mechanical properties of CaPs also limit their applications in orthopaedics. To overcome the weakness of CaPs, a broad range of materials have

been proposed to reinforce CaPs, including carbon nanotubes (CNTs) (Chew *et al.*, 2011; Low *et al.*, 2011), polymers (Rezwan *et al.*, 2006; Neumamn and Epple, 2006; Yunos *et al.*, 2008) such as chitosan (Xu and Simon, 2005), poly(D,L-lactic-co-glycolic acid) (PLGA) (Durucan and Brown, 2000) and poly(lactide acid) (PLA) (Mickiewicz *et al.*, 2002). However, the research related to nanoshell based CaPs is rarely reported.

Nanoshells can be defined as nanoscale structures containing a rigid shell surrounding a solid and/or liquid core (template) composed of a different material. Nanoshells are typically in the size range of 20 nm to 200 nm (Sounderya and Zhang 2008). Their sizes are small enough to make them ideal for targeted injection to specific zones of the body. Nanoshells can enhance the thermal and chemical stability, improve solubility and have less cytotoxic (Ishikawa, 2003; Dorozhkin, 2008; Sounderya and Zhang 2008). Various synthesis routes have been developed over the past few years to prepare nanoshells such as interfacial polymerisation (Scher *et al.*, 1998; Vincent, 2006), layer-by-layer deposition (Ai *et al.*, 2003; Vincent, 2006), sol-gel method (Pal and Chakravorty, 2005; Chatterjee, *et al.*, 2005; Basu and Chakravorty, 2006) and others. The great challenge in this research field is to develop synthesis method that can control the particle size and shape uniformly (Hu *et al.*, 2010). Nanoshells have a plethora of applications related with them. For instance, they are used in imaging cancer cells and other therapeutic applications (Kalele *et al.*, 2006). In this project, the idea of nanoshells is expected to improve the drawback of CaPs.

2.2 Calcium Phosphate (CaP) Based Bone Biomaterials

Bone is organic-inorganic hybrid composite of protein and mineral with superior strength, hardness and fracture toughness (Gao *et al*., 2003). Bone can be categorised as short, flat, and tubular. The function of the bone is to mainly withstand the forces imposed by normal activities (Shi, 2006).

In Europe, USA and Japan, osteoporosis perhaps affects 75 million people (European Foundation for Osteoporosis and The National Osteoporosis Foundation; 1997). It is also estimated that over 1 million people in Malaysia are at risk of osteoporosis (Arthritis Foundation Malaysia, 2011). The biggest problem created by osteoporosis, one of the bone diseases, is fractures, which may be the first noticeable symptom of disease in patients. The problem becomes a chronic burden on individuals and even the public. The risk of fracture increases dramatically with age in both sexes. This may be due to bones become more fragile and the risk of falling increases (Office of Surgeon General, 2004). In recent years, there has been considerable progress in understanding bone biomaterials (e.g., bone substitutes). This has noteworthy implications for the future management of bone loss (Chow, 2009).

The attempt to discover substitutions for repair of fatally damaged human bones dated back to centuries (Katti, 2004). The common principle for materials selection in the finding of new bone biomaterials are biocompatibility and mechanical performance. Metals usually show excellent mechanical properties but have poor biocompatibility at the same time, causing stress shielding and release of unsafe metal ions, and thus resulting ultimate failure and removal of implant. In

general, polymeric materials alone tend to be too weak to be suitable for meeting the condition of stress deformation responses in human bone such as total hip substitution components. Ceramics were investigated as bone substitute biomaterials owing to their simplicity of processing and forming, good biocompatibility, mechanical strength and toughness (Katti, 2004). Because of lack of chemical bonding between conventional ceramics such as sintered Al_2O_3 and tissue, their applications as a potential bone substitute are restricted (LeGeros, 2008). Since the composite materials with engineered interfaces resulting in combination of greater biocompatibility and mechanical performance, the focuses of many existing researches are composite materials such as CaP based composites (Katti, 2004).

CaPs have been extensively investigated due to the similarities with the bone mineral and their structural and surface features are accountable for their superior biocompatible as well as bioactive properties, has the ability to interact with the biological milieu to enhance the biological response (Navarro *et al.*, 2008). Moreover, the CaP biomaterials are osteoconductive materials that only allow the formation of bone on their surface by serving as a scaffold. In patients with diminished bone forming ability, bone tissues do not necessarily bond to CaP biomaterials at a clinically satisfactory level. Moreover, the osteoblasts are the cells responsible for the growth of the bone matrix and are found on the developing bone surface. If the CaP biomaterials possess osteoconductive and osteoblasts properties, the expansion of clinical application for the CaP biomaterials is a certain (Salata, 2004; Ito and Ohgushi, 2005).

Another important property of bone is the osteoinductivity that allows the bone to repair and regenerate itself. However, CaP biomaterials are commonly known to be osteoconductive but not osteoinductive (LeGeros, 1991; LeGeros, 2008). Osteoinductive properties can only be introduced to CaP biomaterials by designing the CaPs with appropriate geometry, topography, combined appropriate macroporosity/microporosity and concavities that will allow the entrapment and concentration of circulating growth factors or osteoprogenitor cells responsible for bone formation or combining CaP with growth factors or bioactive proteins (LeGeros, 1991).

2.3 Types of Calcium Phosphate (CaP) Biomaterials

There are many types of CaP biomaterials in clinical applications including CaP ceramics, calcium phosphate cements (CPCs) and CaP based composites (LeGeros, 2008). All these CaP biomaterials are described with a brief introduction to help understanding of CaP biomaterials.

2.3.1 Calcium Phosphate (CaP) Ceramics

A variety of dense and porous CaP ceramics have been developed in various forms including HA from synthetic or natural (from coral), calcium-deficient hydroxyapatite (CDHA), TCP (α-tricalcium phosphate (α-TCP) and β-tricalcium phosphate (β-TCP)), biphasic calcium phosphate (BCP), tetracalcium phosphate (TTCP) and amorphous calcium phosphate (ACP) (Fernández, 1999a; Fernández, 1999b; Dorozhkin, 2009a). The properties of these CaP ceramics are listed in Table 2.1. CaP ceramics in blocks or granules are the main raw materials used for bone substitutes (Suchanek and Yoshimura, 1998). These forms are not suitable when

cavities are not straightforwardly accessible or when it would be preferable to carry out microinvasive percutaneous surgery (Low *et al.*, 2010).

Table 2.1: The properties of various phases of CaP ceramics.

CaP ceramics	Chemical formula	Ca/P ratio	pH stability range in aqueous solutions at 25°C	References
Amorphous calcium phosphate (ACP)	$Ca_x(PO_4)_y.nH_2O$, n=3-4.5; 15-20% H_2O	1.20-2.20	~5-12	Dorozhkin, 2009a
α-tricalcium phosphate (α-TCP)	$Ca_3(PO_4)_2$	1.50	[a]	Fernández, 1999a; Dorozhkin, 2009a
β-tricalcium phosphate (β-TCP)	$Ca_3(PO_4)_2$	1.50	[a]	Fernández, 1999a; Dorozhkin, 2009a
Calcium-deficient hydroxyapatite (CDHA)	$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$; (0<x<1)	1.50-1.67	6.5-9.5	Fernández, 1999b; Dorozhkin, 2009a
Hydroxyapatite (HA)	$Ca_{10}(PO_4)_6(OH)_2$	1.67	9.5-12	Fernández, 1999a; Dorozhkin, 2009a
Tetracalcium phosphate (TTCP)	$Ca_4(PO_4)_2O$	2.00	[a]	Fernández, 1999a; Dorozhkin, 2009a

Note: [a] These compounds cannot be precipitated from aqueous solutions.

The ACP phase is an intermediate phase in the preparation of a number of CaPs. ACP occurs in many biological systems, particularly in primitive organisms, where it is believed to provide a reservoir of Ca^{2+} and PO_4^{3-}. ACP is straightforwardly converted into poorly crystalline apatite comparable to bone mineral crystals and benefit can be taken of its high reactivity to produce bioactive biomaterials (Combes and Rey, 2010). ACP plays a crucial role in the

biomineralisation of bone as it is a precursor to crystalline bone apatite (Li *et al.*, 2007). Moreover, ACP is extensively used as a precursor to prepare crystalline CaPs with different compositions (Layrolle *et al.*, 1998). ACP based biomaterials are used in the form of coatings, cements, ceramics or composites (see Table 2.2). The instability of ACP raises issues for mass production, storage and processing that limit the improvement of ACP based biomaterials (Combes and Rey, 2010).

Table 2.2: ACP based biomaterials (Combes and Rey, 2010).

Type of ACP based biomaterials	Applications	Main CaP-related effects
Ionic cements	Bone substitute Dental applications	Active hardening agents Bioresorbable surface reactivity Provider of Ca^{2+} and PO_4^{3-} ions
Coatings	Coating of metallic prostheses	Biodegradable and reactive coating
Mineral-organic composites	Teeth, enamel remineralisation Bone substitute	Mechanical properties Ca and PO_4 release in relation with biological activity

HA is a bioactive ceramics commonly used as powders or in particulate forms as coatings for metallic prostheses to enhance their biological properties (Liu *et al.*, 2001). In addition, HA has also been used for a range of biomedical applications such as bone tissue regeneration, cell proliferation, and drug delivery (Sopyan *et al.*, 2007). HA is the ideal phase for use inside human body as it has outstanding stability above pH 4.30 (human blood pH being 7.30) and can show strong relation to host hard tissues owing to the chemical similarity between HA and mineralised bone of human tissue (Kalita *et al.*, 2007). However, HA remains in the human body for a long time after implantation (Kamitakahara *et al.*, 2008).

Moreover, the mechanical properties of HA is very poor as compared to human bone. Meanwhile, the bone mineral present a greater bioactivity as compared to HA (Kalita *et al.*, 2007).

As a first approximation, CDHA similar to bone mineral and may be considered as HA although lacking the ionic substitutions (Brown and Martin, 1999; Dorozhkin, 2009a). It is a poorly crystalline material with a ratio of calcium-to-phosphorus (Ca/P) varying between 1.50 and 1.67 (Mickiewicz, 2001). The structure contains vacant Ca^{2+} and hydroxide ion (OH^-) sites, whereas some of the phosphate ion (PO_4^{3-}) are either protonated or substituted with other ions (Boanini *et al.*, 2010). Because of a lack of stoichiometry, CDHA often occurs with ionic substitutions (Dorozhkin and Epple, 2002). The extent depends on the counter-ions of the chemicals used for preparation. Direct determinations of the CDHA structures are still missing and the unit cell parameters remain uncertain. The ion substituted CDHA, such as sodium ion (Na^+) for Ca^{2+} with some water forms biological apatite addition which is the main inorganic part of animal and human normal and pathological calcifications (LeGeros, 1991; Rey *et al.*, 2006; O'Neill, 2007). Hence, CDHA is a very promising compound for industrial manufacturing of synthetic bone substitutes.

One might expect that implanted materials should exhibit resorbable property through bone regeneration, followed by complete substitution for the natural bone tissue after stimulation of bone formation. Therefore, for bone regeneration, much attention has been paid to TCP as scaffold materials (Kamitakahara *et al.*, 2008). It has been proved to be resorbable *in vivo* with new

bone growth replacing the implanted TCP (Gibson *et al.*, 2000). The two forms of TCP that are known to exist are α-TCP and β-TCP. β-TCP transforms into a high-temperature phase, α-TCP at temperatures above 1125 ºC. At room temperature, β-TCP is more stable than the α-TCP. In addition, β-TCP as stable phase is less soluble in water than α-TCP (Yin *et al.*, 2003). Thus, α-TCP has received very little attention in the field of biomedical application. The drawback for using α-TCP is its speedy resorption rate in which limits its usage in this area (Metsger *et al.*, 1999). In contrast, β-TCP is basically a gradually degrading bioresorbable CaP ceramic (Driessens *et al.*, 1978). Therefore, it is a promising material in the field of biomedical applications. It has also been observed to have considerable biological affinity as well as activity and responds very well to the physiological environments (Kivrak and Tas, 1998). These factors give β-TCP an edge over other biomedical materials when it comes to resorbability and substitution of the implanted TCP *in vivo* by the new bone tissue (Gibson *et al.*, 2000). It is reported that the resorbability of β-TCP *in vivo* might be strongly associated to the characterisation and stability of the β-TCP structure (Okazaki and Sato, 1990; Kalita *et al.*, 2007).

A bioactive idea developed for BCP ceramics. The idea is based on an optimal balance of the more stable phase of HA and more soluble TCP (Daculsi, 1998). Daculsi (1998) prepared BCP macroporous ceramics consisting of a β-TCP and HA with dissimilar β-TCP/HA mass ratios, and implanted them in osseous defects in dogs. BCP ceramics have been considered to be a promising scaffold for utilise with tissue engineering strategies for bulky bone defect reconstruction. BCP ceramics change according to their chemical composition and physical structures, which in conjunction with the implantation site, form (e.g., granules, blocks and

customised pieces) and the intrinsic conditions of the patient, can give rise to dissimilar rates and patterns of human bone development (Lobo and Arinzeh, 2010). The resorbability of BCP ceramics was enhanced with raising the β-TCP/HA mass ratio. They remarked the formation of bone-like apatite crystals on the BCP ceramics surfaces, which was associated with the β-TCP/HA ratios of the BCP ceramics. They hypothesised that the formation of the bone-like apatite may be owing to the precipitation of Ca^{2+} and PO_4^{3-} released from the β-TCP component in the BCP ceramics. In order to apply suitable BCP to meet specific biological needs, it is essential to control the BCP ceramics by altering their β-TCP/HA ratios (Cho *et al.*, 2010). However, it also proposed that the combination of β-TCP with HA may lead to more complexes biological and chemical incidents caused by both β-TCP and HA (Kamitakahara *et al.*, 2008). The knowledge of such parameters is necessary in choosing a BCP for a particular application (Lobo and Arinzeh, 2010).

For TTCP, its solubility in water is higher as compared to that of HA (Dorozhkin, 2007). TTCP cannot be precipitated from liquid solutions. Therefore, it can only be prepared by a solid-state reaction above 1300 °C. It is very unstable in liquid solutions and it gradually hydrolyses to HA and calcium hydroxide (CaOH) (Dorozhkin, 2009a). As a result, TTCP has never been found in biological calcifications. TTCP is usually used in medicine for the forming of various self-setting cements. Nonetheless, the synthesis and applications of TTCP in nanoscale have not much been reported (Kalita *et al.*, 2007).

2.3.2 Calcium Phosphate Cements (CPCs)

CPCs are injectable paste-like materials that harden in the human body (Ito and Ohgushi, 2005). CPCs consisting of mixtures of different CaP phases, such as β-TCP, TTCP, monocalcium phosphate monohydrate (MCPM), dicalcium phosphate dehydrate (DCPD or brushite), dicalcium phosphate anhydrous (DCPA or monetite) and octacalcium phosphate (OCP). They are mixed with water in a liquid-to powder (L/P) ratio of 1:4 to form a paste that can be conventional to osseous defects with complex shapes and set *in vivo* to form HA with tremendous osteoconductivity without any acidic or basic by-product (Brown and Chow, 1985; Bai *et al.*, 1999). The improvement of self-setting CPCs has extended the use of CaPs to injectable bone substitutes that can be moulded and shaped to fit irregular defects, and reveal osteo-integrative properties similar to or better than those of bulk CaPs (Brown and Chow, 1985). They had been chosen for clinical use because of their suitability for repair, augmentation, regeneration of bones and the advantages related self-hardening properties of the cements (Chow, 2009).

Table 2.3 lists the properties of three common formulations of CPCs (Schmitz *et al.*, 1999). In a nutshell, the advantages of CPCs include being injectable, moldable, to adapt to the human bone defects, to exhibit excellent biocompatibility and to be osteoconductive. CPCs also have their weakness in modestly invasive clinical applications, in which is their low capability to be injected through a thin long cannula attached to a syringe (Khairoun *et al.*, 1998; Leroux *et al.*, 1999; Bohner and Baroud; 2005; Low *et al.*, 2010). Research efforts on CPC have been somewhat unfocused so that despite a wealth of knowledge gained, clinical applications of CPC remain limited to a relatively narrow area (Chow, 2009).

Table 2.3: Properties of CPCs (Schmitz *et al.*, 1999).

Formulation	Bone Source	α-BSM Embarc	Norian SRS/CRS
Components	TTCP and DCPD	Decarbonated ACP and either DCPD, calcium metaphosphate, calcium heptaphosphate, calcium pyrophosphate, or TCP	Monocalcium phosphate, α-TCP, calcium carbonate
Compressive strength	36 MPa (for first 24 h)	Unknown	55 MPa
Resorbable	Minimally	Yes	Completely
Commercially available	Yes	Yes	Yes
Pore diameter	2 – 5 nm	Unknown	300 Å
Initial setting time	10 – 15 min	15 -20 min	10 min
Final setting time	4 h	1 h	12 h
Osteoconductive	Yes	Yes	Yes
Sets in presence of fluid	No (must be kept dry)	Yes	Yes

2.3.3 Calcium Phosphate (CaP) Composites

The goal for development of composite materials has been achieved by a combination of properties of various materials which not achievable by any of the elemental materials acting alone (Göller *et al.*, 2003). Thus, CaP is commonly used in combination with various materials including CNTs (Chew *et al.*, 2011; Low *et al.*, 2011), polymers (Rezwan *et al.*, 2006; Neumamn and Epple, 2006; Yunos *et al.*, 2008) such as chitosan (Xu and Simon, 2005), PLGA (Durucan and Brown, 2000) and PLA (Mickiewicz *et al.*, 2002) as composites to overcome their limitations had been discussed in previous section. CaP composites described here have distinctive features (Ito and Ohgushi, 2005).

Ever since the discovery of CNTs by Iijima (1991), a growing attention in the applications of CNTs has been focused on their use as reinforcement in dissimilar matrix materials due to their outstanding mechanical performance (Treacy *et al.*, 1996; Low *et al.*, 2010; Chew *et al.*, 2011; Low *et al.*, 2011). CNTs are being investigated for biomedical applications, such as neural implants and tissue scaffolds, which utilise their high tensile strength, chemical stability and electrical conductivity (Mattson *et al.*, 2000; Correa-Duarte *et al.*, 2004; Gheith *et al.*, 2005; Boccaccini and Gerhardt, 2010). Thus, CNTs could be an attractive reinforcement for CaP biomaterials.

There is also an interest in developing CaP composites reinforced by multi-walled CNTs (MWCNTs) and bovine serum albumin (BSA) (Chew *et al.*, 2011; Low *et al.*, 2011) in order to improve the mechanical properties of CaPs for applications as injectable bone substitutes. At appropriate amounts of BSA, such as low concentration, BSAs are able of promoting CaPs crystal growth. This is due to BSA could stabilise nuclei and encourage growth of CaP crystals at low concentrations of BSA (Low *et al.*, 2011). However, crystal growth seems to be slowed down by high BSA coverage at higher concentration (Combes *et al.*, 1999). The results of the characterisations of the compressive strength for the pure CPC, CPC incorporated with three dissimilar types of MWCNTs (e.g., Pristine MWCNTs, hydroxylated MWCNTs (MWCNTs–OH) and carboxylated MWCNTs (MWCNTs–COOH)) and BSA are shown in Figure 2.1. From the results of the characterisations of the compressive strength, the CPC/MWCNTs–OH/BSA composite showed significantly enhanced compressive strength (≈16 MPa) if compared to pure CPC (≈1 MPa) (Chew *et al.*, 2011).

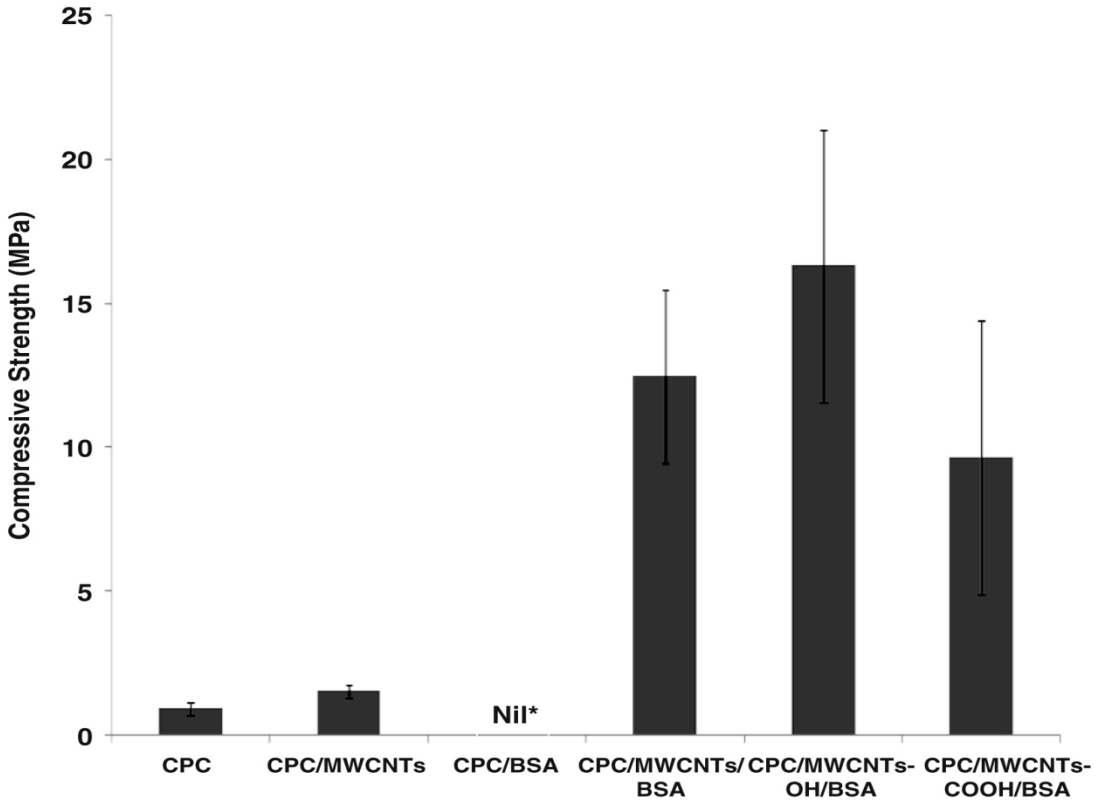

Figure 2.1: The comparison of the compressive strength values of the pure CPC, CPC/ MWCNTs, CPC/BSA, CPC/MWCNTs/BSA, CPC/MWCNTs–OH/BSA, and CPC/MWCNTs–COOH/BSA composites. *Note that the compressive strength of the CPC/BSA composite could not be measured due to the composite was too weak to form the required shape for compressive characterisation purposes (Chew *et al.*, 2011).

Because of the uncertainties about the biocompatibility and toxicity of CNTs for clinical applications (Boccaccini and Gerhardt, 2010), further study should focus on the *in vitro* cell biological investigations and on the *in vivo* performance of the CaP composites that contain CNTs (Chew *et al.*, 2011).

Besides, polymers are materials that can be used for multipurpose. They are being enthusiastically investigated as materials for medical applications (Mickiewicz, 2001). In addition, the combination of CaPs with polymers can enhance the mechanical properties of the composite (Durucan and Brown, 2000; Mickiewicz *et al.*, 2002; Xu and Simon, 2005; Rezwan *et al.*, 2006). For example, Durucan and

Brown (2000) made HA–PLGA composite with a tensile strength of 13.3 ± 0.9 MPa, a flexural strength of 24.8 ± 1.7 MPa, and Young's modulus of 2.8 ± 0.3 GPa, which showed a modest enhancement over the pure material. Moreover, the polymer addition might have other functions, such as biodegradable polymer can act as carrier for biomolecules, growth factors and antibiotics, hence, rising the capability of tissue engineering construct (Yunos *et al.,* 2008). However, they show bulk degradation, leading to a loss in mechanical properties and they reduce the effect of stress-shielding. Thus, they are too weak to be used in most of the load bearing situations, and are only recommended in certain clinical indications, such as elbow and ankle fractures (Hofmann, 1995; Athanasiou *et al.,* 1996). Since 1960s, some of the most popular synthetic polymers (e.g., PLA, PLGA) have been extensively studied and approved by Food and Drug Administration (FDA) in clinical application as degradable sutures (Li *et al.,* 1990; Behravesh *et al.,* 1999), however, not all polymers used are biodegradable biomaterials. It would be vital to examine the biocompatibility, toxicity and behaviour of the CaP composites both *in vitro* and *in vivo* (Mickiewicz, 2001).

In short, a combination of two materials draws on the advantages of each to make an advanced composite biomaterial (Mickiewicz *et al.*, 2002). However, the drawbacks of the existing reinforced materials cannot be neglected.

2.4 Nanoshells

Since nanomaterials are at the leading edge of the speedily developing field of nanotechnology, the unique size-dependent properties of nanomaterials make them superior and essential in many fields. As a result of their outstanding and