IN SITU SYNTHESIS AND CHARACTERISATION OF CHITOSAN/NANOHYDROXYAPATITE COMPOSITE

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ABSTRACT

This study aimed to synthesize and characterise the homogenous chitosan/hydroxyapatite (CS/HA) composite by *in situ* precipitation method. The optimum ratio of chitosan/hydroxyapatite composite was studied by varying the concentration of chitosan, orthophosphoric acid (H_3PO_4) and calcium nitrate ($Ca(NO_3)_2.4H_2O$) used in this formulation. The CS-H_3PO_4 solution was prepared and reacted with $Ca(NO_3)_2.4H_2O$ to produce CS/HA composites ratio of 30/70, 40/60 and 45/55, which were identified by thermogravimetric analysis. Fourier transform infrared spectroscopy results showed absorption peaks at 601.8 cm^{-1} and 562.0 cm^{-1} to 564.2 cm^{-1} which proved the presence of phosphate, PO_4^{3-} of HA component whereas absorption peak at 1591.0 cm^{-1} was corresponding to amide of the chitosan group. Scanning electron microscopy analysis on the composite surface revealed that CS/HA composite with 45/55 ratio exhibited particles with needle like shape with cross-section of 5.41 nm and length of 37.2 nm distributed in the matrix. On the other hand, composite with ratio of 30/70 and 40/60 were more rounded and less uniformly dispersed. The compression strength of 45/55 composite was the highest as compared to 30/70 and 40/60 composite. X-ray powder diffraction result showed similar pattern for all samples, of which chitosan appeared at $2\theta = 10.6^o$ and 20.4^o while crystalline peaks of HA at $2\theta = 25.8^o$ and 40.0^o. Bioactivity study proved that mineralization was occurred on the surface of all samples, but it was more significant in 30/70 composite sample. Biocompatibility study revealed that all composites have no indication of cell toxicity. The results showed that the simple *in situ* precipitation technique successfully developed CS/HA composite with good mechanical properties and biocompatibility, which is suitable for bone filler application.

SINTESIS *IN SITU* DAN PENCIRIAN KOMPOSIT KITOSAN/NANOHIDROKSIAPATIT.

ABSTRAK

Kajian ini bertujuan mensintesis dan mencirikan komposit kitosan/hidroksiapatit (CS/HA) homogen melalui kaedah pemendakan *in situ*. Nisbah optimum kitosan/hidroksiapatit telah dikaji dengan mengubah kepekatan kitosan, asid ortofosforik (H_3PO_4) dan kalsium nitrat ($Ca(NO_3)_2.4H_2O$) yang digunakan dalam formulasi ini. Larutan CS-H_3PO_4 disediakan dan bertindak balas dengan $Ca(NO_3)_2.4H_2O$ untuk menghasilkan komposit CS/HA dengan nisbah peratus 30/70, 40/60 dan 45/55 ditentukan dengan menggunakan alat analisis termogravimetri. Keputusan spektroskopi perubahan cahaya merah Fourier menunjukkan puncak penyerapan pada nombor gelombang 601.8 cm^{-1} dan pada 562.0 cm^{-1} sehingga 564.2 cm^{-1} yang membuktikan kehadiran kumpulan fosfat, PO_4^{3-} daripada HA manakala puncak penyerapan pada 1591.0 cm^{-1} menunjukkan kehadiran kumpulan amida dalam kitosan. Analisis mikroskop imbasan elektron di permukaan komposit CS/HA pada nisbah 45/55 menunjukkan struktur zarah berbentuk seakan-akan jarum dengan keratan rentas 5.41 nm dan panjang 37.2 nm yang tersebar dalam matrik. Manakala, zarah hablur dalam komposit dengan nisbah 30/70 dan 40/60 mempunyai struktur lebih bulat dan kurang seragam. Kekuatan mampatan untuk komposit 45/55 adalah yang tertinggi berbanding dengan komposit 30/70 dan 40/60. Keputusan pembelauan sinar X menunjukkan corak yang sama untuk semua sampel yang mana kitosan hadir pada puncak $2\theta = 10.6^o$ dan 20.4^o manakala puncak kristal HA ialah pada $2\theta = 25.8^o$ dan 40.0^o. Kajian bioaktiviti telah membuktikan terjadinya mineralisasi pada permukaan kesemua sampel, tetapi ia lebih banyak hadir pada sampel komposit 30/70. Kajian biokompatibiliti mendedahkan bahawa kesemua komposit tidak mempunyai tanda-tanda ketoksikan sel. Keputusan kajian menunjukkan teknik pemendakan mudah *in situ* berjaya menghasilkan komposit CS/HA dengan ciri-ciri mekanikal dan biokompatibiliti yang bagus, dan sesuai digunakan dalam aplikasi pengisi tulang.

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols and Abbreviation

$^{\circ}C$	Celsius
µm	mikrometer
AFWSLMWC	Acid-Free-Water-Soluble Low-Molecular-Weight Chitosan
ATR	Attenuated Total Reflection
BSE	Back Scattered Electrons
C1	Carbon (First)
C2	Carbon (Second)
$Ca(NO_3)_2.4H_2O$	Calcium Nitrate
Ca/P	Calcium/Phosphorus
$Ca_{10}(PO_4)_6(OH)_2$	Hydroxyapatite
$CaCO_3$	Calcium Carbonate
CaO	Calcium Oxide
CC_{50}	Cytotoxicity concentration 50%
cm^3	centimeter square
CO	Carbonate
CS	Chitosan
CS/HA	Chitosan/Hydroxyapatite
CS–$CaCO_3$	Chitosan–Calcium Carbonate
DD	Degree of Deacytelation
DNA	Deoxyribonucleic Acid
ECM	Extra Cellular Matrix

EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectrometer
GAG	Glycosaminoglycan
GBMCs	Goat Bone Marrow Stromal Cell
GlcN	2-amino-2-desoxy-b-D-glycopyranose
GLcNac	1,4-*N*-acetyl-D-glucosamine
H	Hydrogen
H_2O	Water
H_3PO_4	Orthophosphoric Acid
HA	Hydroxyapatite
He	Helium
IBP	Internal Bubbling Process
kN	kiloNewton
kV	kiloVolt
LMWC	Low-Molecular Weight Chitosan
LPS	Lipopolysaccharide
mA	milliAmpere
Mg	Magnesium
min	minute
mM	millimolar
mm	millimeter
MNac	N-acetyl-muraminic acid
MPa	Megapascal
N	Nitrogen

NaOH	Natrium Hydroxide
NH_2	Amide
NH_4HCO_3	Ammonium Hydrogen Carbonate
nHA	Nanohydroxyapatite
nm	nanometer
O	Oxygen
OH	Hidroxide
pK_a	acid dissociation constant
PMMA	Polymethyl Methacrylate
PO_4	Phosphate
s	second
SBF	Simulated Body Fluid
SE	Secondary Electron
SEM	Scanning electron microscope
siRNA	small interfering Ribonucleic Acid
TCP	Tricalcium Phosphate
TGA	Thermogravimetric analysis
TGF-β1	Transforming Growth Factor-β1
v/v	volume per volume
W	Watts
w/v	weight per volume
wt%	weight percent
XRD	X-ray diffractometer
θ	Theta

CHAPTER 1

INTRODUCTION

1.1 Research Background

All over the world people suffered from bone defects caused by loss, infection and tumor which required prompt action in orthopedic surgery. Researchers successfully developed bone substitute to maintain and restore the function of bone tissue. The most effective approaches is to develop scaffold tissue implant for bone regenerating template in order to induce the growth of new bone tissues. Advanced bioceramic such as hydroxyapatite (HA) has been introduced as a potential material for bone substitution because of it biocompatibility, light weight, ductility and moldability.

Commercial available synthetic hydroxyapatite (HA) ($Ca_{10}(PO_4)_6(OH)_2$) is a bioactive ceramic material and resembles to natural bone and teeth (Murugan & Ramakrishna, 2005). Hydroxyapatite is not just bioactive, but also osteoconductive, nontoxic, biocompatibility, non-immunogenic and crystallographically similar to bone mineral (Aoki, 1991). Synthetic HA however is an inert material, thus it has very low biodegradability in the body. This drawback effect the function of HA as bone substitute as the permanent present of this materials in body subsequently will impede the total growth of new bones. Furthermore, HA is very high crystalline materials, therefore it is brittle, low elasticity and low stiffness and durability (Kim et al., 2004). The combination of HA with polymers is a possible method to overcome the low degradability of HA and at the same time increase the elasticity of the material.

The potential biopolymer such as collagen, gelatin, alginate, polyesters and ploymethyl-methacrylate (PMMA) has been used to fabricate HA composites (Neumann & Epple, 2006).

In this study, chitosan will be used as a polymer to develop chitosan/HA composites. The advantages of chitosan are non-toxic, biocompatible, biodegradable and antibacterial activity. Chitosan can be process to gels (Madhumathi et al., 2009), membranes (Madhumathi et al., 2009), nano fibers (Shalumon et al., 2009), beads (Davidenko et al., 2010), nano particles (Anitha et al., 2009), scaffolds (Madhumathi et al., 2009) and sponge forms (Portero et al., 2007).

Chitosan/hydroxyapatite composites have been developed by many researchers due to successful in term of mechanical properties, good cell growth and biocompatibility (Danilchenko et al., 2009; Nikpour et al., 2012; Zhang et al., 2012; Rogina et al., 2013). Chitosan/hydroxyapatite composites can be synthesized to form powder, membrane, pastes, cements and micro sphere. Nano hydroxyapatite (nHA) has been found to enhance the growth of apatite on the composite scaffolds to produce a potential implant (Neumann & Epple, 2006).

Chitosan/hydroxyapatite composite has been synthesized by mechanical mixing of chitosan and HA. However, due to wetting problems, the HA was not well distributed in the chitosan matrix. Therefore in this study *in situ* synthesis of HA in the chitosan matrix has been studied.

1.2 Problem Statement

Bone deficiency triggered by tumor, trauma and bone abnormalities is a major problem for human and required surgery to provide skeletal continuity, mechanical support and bone regeneration. Bone does not heal by itself for severe defects, therefore bone grafting is urgently needed to restore the function without jeopardy living tissues. A few techniques available for treatments bone defects included autograft and allograft but the problems happened when the supply of bones are insufficient. Autograft involves bone or tissue implant within the body, while allograft means a donor from individual into another person which is non-identical genetic composition. Sometimes problem occurred when allograft techniques might transfer the pathogen. It is needed amount of

time to care, sterile and proper action on the subject. The alternative ways have been introduced for the last decade. One of the most popular methods is using synthetic biomaterial like hydroxyapatite which is capable to improve several cases associated with autogenous and allogeneic bones. In recent years, the capability of synthetic biomaterial has different characteristics from the natural bone, some of them alleviate the bone strength, good indicator for in vivo analysis, cytotoxicity and rejuvenation of bone tissue.

One of the promising candidate for bone filler is composite of chitosan and nanohydroxyapatite. Chitosan and nanohydroxyapatite biocomposite can be fabricated by physical mixing of chitosan and nanohydroxyapatite. However, one of the major problem is difficult to obtain homogenous mixture of chitosan and nano hydroxyapatite (Li, 2010). Based on this situation, *in situ* chemical precipitation technique was selected to overcome this problems. *In situ* chemical precipitations technique was also identified to be an easiest method and inexpensive to produce CS/nHA composites. Besides that, it is also probably one of the fastest synthesis method to produce large amount of CS/nHA composite products.

1.3 Objectives

The main objectives of this study are:

i. To synthesis chitosan/nanohydroxyapatite composites by *in situ* precipitation technique.

ii. To determine the optimum formulation to produce chitosan/nanohydroxyapatite composite by varying the concentration of chitosan and hydroxyapatite.

iii. To characterize the mechanical and biocompatibility activities of chitosan/nanohydroxyapatite composite.

1.4 Significance of Study

The demand of synthetic bone is increasing every year due to accident cases, traumatic and bone diseases. Production of synthetic HA able to fulfill the demand of bone replacement. The aim of this study is to develop technique significantly and perfect weight ratio of chitosan and hydroxyapatite composite in order to create bone substituted for seed into human bone. Bone is the important rigid organ purposely design for protection, movement and supports of the organs such as skull shields the brain and ribs keep the heart and lungs stability. When diseases inflicted by infection, skeletal cracking, calcium deficiency or bone cancer, it is emerging a critical issue for every person. Focus on advancing biocomposite in bone regeneration is comprehensively needed for longevity of human life.

Bone is a perfect example of biocomposite because consist of protein and mineral at the nanoscale level. Hence, this study provides an identical composite with mixing two heterogeneous biomaterials. CS and HA have been introduced in this study as the individual different type of biomaterials. CS has been treated on wound healing, repair dermal tissue, generation nerve tissue and osteogenic bone tissue. Thus, CS is well known in the biological field in tissue engineering. While hydroxyapatite is inorganic phase in bone tissue. Synthetic hydroxyapatite is produced by chemical synthesis technique which is HA that has similar chemical structure to bone but very

low degradable properties. The growth of new bone therefore impede due to slow degradable of HA template.

The fabrication of chitosan/hydroxyapatite composites fit into all the requirements to induce bone rejuvenation and bone durability. These criterions play a key role to investigate either it is suitable to place into the bone. The homogenous composition must achieve to form a biocompatible composite. Besides, the synthesis technique, nano size HA increase the interaction of composite for cell growth. Furthermore nano particle composites produced high porosity which is important characteristic to improve cell proliferation. The method used in this study is easy to control the required composition ratio between chitosan and HA by varying the composition of chemicals used.

Low processing temperature method used in this study will increase the possibility to incorporate the cell in the mixture when it is required in the future.

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CHAPTER 2

LITERATURE REVIEW

2.1 Chitosan (CS)

Chitin, a poly-beta-1,4-*N*-acetyl-D-glucosamine (GLcNac) is a natural polysaccharide synthesized by the main component of anthropod, crustacean and mollusc in marine life and insects exoskeleton (Khoushab & Yamabhai, 2010). When the number of *N*-acetyl-glucosamine units is higher than 50%, the material is called chitin, but when the number of *N*-glucosamine is lower than 50%, the material is known as chitosan (Khor & Lim, 2003). Chitin is source material for chitosan by deacytelation of chitin under alkaline concentration commonly using sodium hydroxide (Khong et al., 2012) or by enzymatic

hydrolysis (Cai et al., 2006). Chitin and chitosan are more interest value of demand due to their high percentage of nitrogen as well as carbon compared.

2.1.1 Synthesis of Chitosan from Chitin

Chitin is usually obtained from crustacean exoskeleton like crab or shrimp shells and fungal mycelia (Su et al., 1997). Ravi Kumar, (2000) explained that the production of chitosan-glucan complexes was associated with fermentation processes that involved alkali treatment yielding chitosan-glucan complexes. The alkali was prepared to remove the protein and deacetylates chitin simultaneously which depend on the alkali concentration and some soluble glycans were removed (Varki & Marth, 1995). Crustacean shells synthesized in order to remove proteins and the dissolution of calcium carbonate which is present in crab shells in high concentrations. The obtained chitin is deacetylated in 40% sodium hydroxide, NaOH at 120 ^{o}C for 1–3 hours. This process produced 70% deacetylated chitosan as shown in the Figure 2.1.

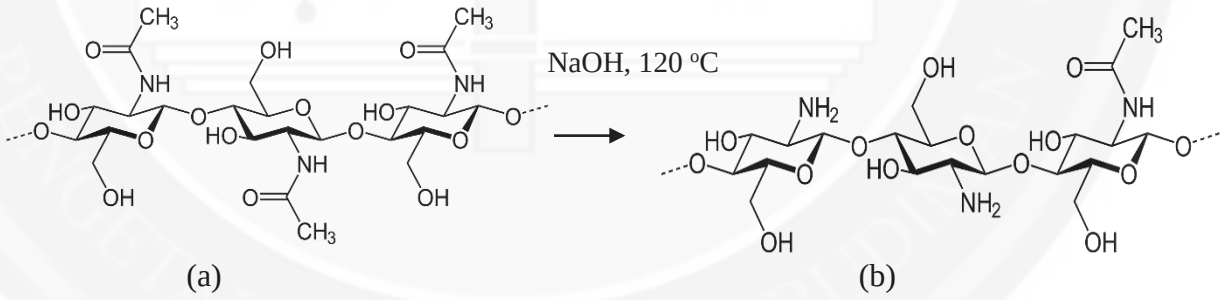

Figure 2.1. Deacytelation of chitin (a) to chitosan (b) in NaOH (Ravi Kumar, 2000)

2.1.2 Chitosan Structure, Properties and Solubility

Chitosan is a semicrystalline polysaccharide, composed of glucosamine and N-acetylglucosamine (Jayakumar et al., 2010). Degree of deacytelation is depending on

the amount of glucosamine and its molecular weight may range from 300 to over 1000 kD with a DD from 30% to 95% (Dornish et al., 2001).

Molecular weight and degree of deacytelation (DD) of chitosan are essential properties and they are affected by conditions during deacytelation process, altercation of post-process by applying physicochemical or enzymatic method. Abdel-Fattah et al., (2007) reported that preparation of different degree of deacytelation can be done by thermochanochemical technique which varied pressure and sodium hydroxide solution. In single crystal of chitosan, Cartier et al., (1990) has been investigated by obtained using fully deacetylated chitin of low molecular weight. The lattice parameters for chitosan are classified into two: hydrated and anhydrous groups.

The structure and properties of chitosan is an important to study in order to diverse its application. Chitosan can be soaked in metal salt solution, inorganic acid solution and organic acid solution. Immersing of chitosan in organic acid solution like formic acid or acetic acid increase its crystallinity (Ogawa et al., 2004). Chitosan with inorganic compounds are influencing cell behaviour producing biomineralization in order to retain growth (Muzzarelli, 2011). Chitosan can be depolymerise, also known as chitosanolysis to produce low-molecular weight chitosan (LMCS), oligosaccharides and monomers (Mourya & Inamdar, 2008). Xing et al., (2005) stated LMCS is more favored because stronger scavenging effect on superoxide and hydroxyl ions which may use in antioxidant. Yue et al., (2008) introduced a new technique to produce acid-free-water-soluble low-molecular-weight chitosan by ozone.

The modification of chitosan is a pivotal derivative for its solubility and widens its application. There are two types of chitosan reactive functional group. First, the free amine group on deacytelated unit and second is both primary and secondary hydroxyl group (Kim et al., 2008). According to Pillai et al., (2009) chemical reaction for the functional group of amino exhibit acetylation, quaternization, reactions with aldehydes and ketones, alkylation, grafting and chelation of metals. While the hydroxyl functional groups also plays a major role in various reactions such as o-acetylation, H-bonding with polar atoms and grafting. These functional groups are modified to improve water solubility, antibacterial and antioxidant properties. The chitosan grafting is a well-known method of chitosan modification. It allows achieving specific purpose such as increasing chelating (Ding, Huang, Li, & Zeng, 2007), bacteriostatic effect (Huang et al., 2007) or enhancing adsorption properties (Cestari et al., 2008). Jeon & Höll, (2003) in their research of modified chitosan through chemical reaction with ethylenediamine showed carbiimide capable to boost the absorption of mercury ions.

Chitosan has poor solubility in water and it make a huge obstacle for fully utilization in many solvents. But chitosan synthesized by the N-deacetylated chitin is soluble in many acidic solvents such as dilute hydrochloric, formic and acetic acids (No et al., 2002). Chitosan is a polyelectrolyte when the existence of acidic solvents that can easily form quaternary nitrogen salts at low pH (Pillai et al., 2009). The solubility of chitosan can be determined by three parameters: degree of deacetylation (DD), distribution of acetyl groups and degree of polymerization (Fan et al., 2009). The backbone branched polysaccharide of chitosan showed good water solubility and altered chemical and biological functions. Morimoto et al., (2011) reported the reductive N-alkylation method by incorporated both high molecular weight chitosan