SYNTHESIS, CHARACTERIZATION AND *IN VITRO* RESPONSE OF CHITOSAN HYDROXYAPATITE COMPOSITES DOPED WITH STRONTIUM, MAGNESIUM AND ZINC

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ABSTRACT

The objectives of this study are to synthesize novel chitosan-hydroxyapatite composites doped with strontium, magnesium and zinc *via in situ* co-precipitation method, to investigate the bioactivity, cytotoxicity and antimicrobial properties of the materials. Physicochemical properties of the products obtained were analysed by X-ray powder diffraction (XRD), Fourier transformed infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), differential thermal analysis (DTA), field emission scanning electron microscopy (FESEM) and energy dispersive X-ray spectroscopy (EDX). The calcium release from the samples in simulated body fluid (SBF) were measured using atomic absorption spectroscopy (AAS). The synthesized composite materials were subjected to bioactivity studies in SBF. *In vitro* cytotoxicity studies were performed on the novel composite materials obtained. Antimicrobial activity investigations against *E. coli* were carried out on all the composite samples. Characterization of the composite samples revealed that three novel composites, *viz*; chitosan-hydroxyapatite composite doped with strontium, chitosan-hydroxyapatite composites doped with magnesium and chitosan-hydroxyapatite composite doped with zinc were successfully synthesized. All the composites obtained showed good bioactivity in SBF. The morphologies of the composites obtained by FESEM analysis revealed that they have good apatite forming ability in SBF. The calcium/phosphorous ratio (Ca/P) of the composites calculated after soaking in SBF have values that are comparable to that of natural apatite. Chitosan-hydroxyapatite composites doped with strontium and zinc were found to be non-cytotoxic against the cell culture used. Chitosan-hydroxyapatite composite doped with magnesium was found to be mildly cytotoxic. Chitosan-hydroxyapatite composites doped with strontium and magnesium show no antimicrobial effects against the *E. coli* bacteria. Chitosan-hydroxyapatite composite doped with zinc show substantial antibacterial effect against *E. coli*. The novel composites obtained thus far can be used potentially for bone grafting, bone filler and template for bone tissue engineering.

SINTESIS, PENCIRIAN DAN TINDAK BALAS IN VITRO KOMPOSIT KITOSAN HIDROKSIAPATIT YANG DIDOPKAN DENGAN STRONTIUM, MAGNESIUM DAN ZINK

ABSTRAK

Objektif kajian ini adalah untuk mensintesis komposit kitosan-hidroksiapatit yang didopkan dengan strontium, magnesium dan zink melalui kaedah pemendapan bersama, menyiasat sifat bioaktiviti, keserasian bio dan antimikrob. Sifat fizikokimia produk dianalisis dengan menggunakan teknik pembelauan sinar-X (XRD), spektroskopi inframerah jelmaan Fourier (FTIR), analisis termogravimetri (TGA), analisis pembezaan terma (DTA), mikroskopi imbasan elektron pancaran medan (FESEM) and serakan tenaga sinar-X (EDX). Pembebasan kalsium daripada sampel dalam simulasi cecair badan (SBF) diukur menggunakan spektroskopi penyerapan atom (AAS). Bahan komposit yang dihasilkan dilakukan ujian bioaktiviti dalam cecair simulasi badan (SBF). Ujian sitotoksik *in vitro* telah dijalankan ke atas bahan komposit novel yang diperolehi. Aktiviti antimikrob terhadap *E. coli* telah dijalankan ke atas semua sampel komposit. Pencirian sampel komposit telah mendedahkan tiga komposit novel, iaitu; komposit kitosan-hidroksiapatit didopkan dengan strontium, komposit kitosan-hidroksiapatit didopkan dengan magnesium dan komposit kitosan-hidroksiapatit didopkan dengan zink telah berjaya disintesis. Kesemua komposit yang diperolehi menunjukkan bioaktiviti yang baik di dalam SBF. Morfologi bahan komposit yang diperolehi daripada analisa FESEM mendedahkan bahawa mereka mempunyai keupayaan membentuk apatit yang baik di dalam SBF. Nisbah kalsium/ fosforus (Ca/P) bagi komposit yang dikira selepas direndam di dalam SBF mempunyai nilai yang setara dengan apatit semulajadi. Komposit kitosan-hidroksiapatit yang didopkan dengan strontium dan zink didapati tidak sitotoksik terhadap sel kultur yang digunakan. Komposit kitosan-hidroksiapatit didopkan dengan magnesium didapati sedikit sitotoksik. Komposit kitosan-hidroksiapatit yang didopkan dengan strontium dan magnesium tidak menunjukkan kesan antimikrobial terhadap bakteria *E. coli*. Komposit kitosan-hidroksiapatit didopkan dengan zink menunjukkan kesan anti-bakteria yang besar terhadap *E. coli*. Komposit novel yang diperolehi setakat ini berpotensi untuk digunakan sebagai pencantum tulang, pengisi tulang dan templat untuk kejuruteraan tisu tulang.

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LIST OF ABBBREVIATIONS AND SYSMBOLS

AAS	Atomic absorption spectrophotometry
DTA	Differential thermal analysis
EDX	Energy dispersive x-ray
FTIR	Fourier transform infra-red
SEM	Scanning electron microscopy
TGA	Thermogravimetric analysis
XRD	X-ray diffraction
HA	Calcium hydroxyapatite
SBF	Simulated body fluid
β- tcp	Beta- tricalcium phosphate

β	Beta
cm	Centimetre
m	Meter
nm	Nanometer
M	Molarity
θ	Theta
Å	Armstrong

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Apatites are ceramic biomaterials with a general formula $W_5(YO_4)_3X$, where W is usually a divalent cation (Ca^{2+}, Sr^{2+}, Cd^{2+}, Pb^{2+}, Ba^{2+}, etc.), YO_4 is a trivalent or tetravalent anion (PO_4^{3-}, VO_4^{3-}, AsO_4^{3-}, etc) and X is a monovalent anion (OH^-, F^-, Cl^-, and Br^-) (Badraoui et al., 2009 ; Kanazawa et al., 1991 ; Legeros et al., 1969; Manjubala et al., 2001). Calcium hydroxyapatite, (HA) is the main constituents of mineral phosphate and also a major inorganic constituent of bones and teeth. Among all the calcium phosphate ceramics used as bone grafts and bone replacement biomaterials, HA closely bear a similarity to the main inorganic phase of bones and teeth in humans (Aina et al., 2013; El-Hammari et al., 2008; He et al., 2013). HA are in abundance in both nature and living organisms (Dorozhkin, 2002). HA are of immense importance in numerous areas of research e.g. biomaterials, luminescent materials, ion exchanges, catalysis etc (Bouka et al., 2007; Boukha, Kacimi, Ziyad, Ensuque, & Bozon-Verduraz, 2007; Chang et al., 2010; Kendrick et al., 2007; Zahouily et al., 2005). HA is also used in the treatment of polluted and industrial waste water (Batton et al., 2007; Ribeiro et al., 2006) and as adsorbent for chromatography to separate biomaterials (Suchanek & Yoshimura, 1998).

HA is commonly applied in biomedical field due to it good biocompatibility, osteoconductivity and similarity to the inorganic component of bone tissues (Lin et al., 2013). However, synthetic HA material has the shortcomings of having unsuitable degradation rate, low mechanical strength, and comparatively poor biological properties compared to bone apatite (Kulanthaivel et al., 2015).

Incorporation of HA with chitosan to produce composites in order to improve their solubility and hence bioactivity (Matos et al., 2010; Turki et al., 2013; Webster et al., 2004; Yasukawa et al., 2012) the biocompatibility, degradation rate and bone bonding ability of HA have been extensively investigated (Muzzarelli, 2011; Nikipour et al., 2012; rajkumar et al., 2013; Rogina et al., 2013). Even though the incorporated chitosan has the ability to stimulate the formation of an apatite layer on biomaterials and provide better degradation rate, it has the disadvantage of having low bioactivity and poor mechanical strength.

Incorporation of some elements into the HA biomaterials have also been broadly applied to improve their solubility and hence bioactivity (Matos et al., 2010; Turki et al., 2013; Webster et al., 2004; Yasukawa et al., 2012). Strontium, magnesium, zinc, silicon, and copper among other metals are some of the most effective and common elements reported to have been used to improve the bioactivity of HA biomaterials (Pan et al, 2009; Alshemary, 2015; Kumar, 2012).

For example HA doped with strontium has been shown to improve a number of biological properties, such as solubility of biomaterials (Landi et al., 2007; Pan et al., 2009), antiresorptive activity, osteoblast stimulation, increased cell proliferation (Aina et al., 2013; Lin et al., 2013) and osteoclast apoptosis, thereby encouraging bone growth and formation (Christopher, 1997). Strontium has also been reported to have the potential in the treatment of osteoporosis (Meunier, 2004). In many reports, it has been shown that strontium can readily substitute calcium in the lattice of HA due to it similar charge-to-size ratio with calcium (Bigi et al., 1981; Rokita, 1993)

It has been reported that HA doped with magnesium enhances bone cell adhesion, osteogenic properties, proliferation and mineralization (Farzadi et al., 2014; Šupová, 2015). Other biological effects has been found to improve when magnesium is substituted for calcium in HA. These include solubility, bioactivity and osteoblast stimulation (Nabiyouni et al., 2015). The deficiency of magnesium has been found to cause cessation of bone growth, decreased osteoblastic activity and bone fragility. Magnesium is the most abundant minor element found in bone apatite and can functions as a co-factor for several essential enzymes in *in vi*vo conditions (Kulanthaivel et al., 2015). Because of the association of magnesium with biological apatites, magnesium substituted HA is expected to have good bioactivity and biocompatibility.

Several studies have been carried out on zinc substitution for calcium in the apatite structures and the effects of such substitution on the properties of HA. Zinc has been found to be the most abundant trace metal in bone mineral. It has direct stimulatory effects on osteoblastic cell *in vitro* as well as inhibitory effect on osteoclastic bone resorption *in vivo* (Kumar et al., 2012; Ren et al., 2009) in addition to it antimicrobial activity (Stanić et al., 2010). The gradual release of zinc into the body by zinc incorporated implants have been found to promote bone formation and accelerate the recovery of patients (Li et al., 2008). Zinc substituted HA has been demonstrated by many studies to improve the bioactivity of HA significantly (Kumar et al., 2012; Stanić et al., 2010; Thian et al., 2013).

1.1 Statement of the Problem

The increasing demand for suitable biomaterials for the treatment of bone diseases arising from trauma and tumor, and also for the repair of bone fractures or defects in humans has necessitated the drive for the fabrication of novel implant materials. The regeneration or repair of damaged bone due to diseases or fractures has been a complicated clinical issue in orthopaedic surgery.

The lack of insufficient bone substitute has caused the death of so many people. The use of exogenous and endogenous bone tissue to supplant the lost bone is found to be connected with several issues. The endogenous bone material has limited availability and more so it requires additional surgery. The exogenous bone implants has the disadvantage of being rejected by the human body or disease may be transmitted together with the implants (Suchanek & Yoshimura, 2007). Metallic implants also have the shortcomings of causing chronic inflammation due to corrosion, stress shielding during post healing and fatigue or loosening of implant (Al-Qasas & Rohani, 2005).

HA have been used as suitable substitute for the exogenous and endogenous bone implants for long time bone repair and regeneration due to their biocompatibility, osteoconductivity, non-toxicity and non-inflammatory properties. The most important property of a biomaterial is not only it ostoeconductive and bone binding abilities but also its controllable degradability (Nikpour, 2012).

Synthetic HA on its own cannot be used due to its brittle feature, poor mechanical properties and slow biodegradation rate. To improve the biodegradation rate some researchers have looked at polymeric materials (e.g. polymethyl methacrylate and poly caprolactone) with bioceramic hydroxyapatites (Jongwattanapisan et al., 2011). These polymeric materials have anionic groups such as OH^-, $COOH^-$ and $-NH_2$ which form on the surface of these polymers and the nucleation site of the crystalline HA particles. The disadvantage of this is the breakdown of the biopolymer *in vivo*. Natural chitosan polymer with HA have been used as an alternative route to overcome this issue. Even though the presence of chitosan in the chitosan-HA composite has the ability to stimulate the formation of an apatite layer on biomaterials, its lacks the ability to improve the bioactivity of the composite, hence the need for additional improvement of the composite (Jongwattanapisan et al., 2011).

From the above discussion it is clear that HA alone do not have the brittleness, degradability and mechanical strength to be used as a perfect bone implants materials. Even though the incorporation of chitosan into HA has been found to improve the biodegradability, biocompatibility and antimicrobial properties of the composites, it is also lacking in improving the bioactivity of the material.

Since the incorporation of strontium, magnesium and zinc into the HA biomaterial have been found to improve its solubility (calcium release ability) and bioactivity, enhanced the apatite forming ability on the sample surface, and considerably increased the *in vitro* dissolution of HA (Cacciotti et al., 2009; Matos et al., 2010; Turki et al., 2013; Webster et al., 2004; Akemi et al., 2012; Kumar, 2012), doping chitosan-HA composite with these metals in will have significant effects on the bioactivity of the composite.

This research work was aimed at doping chitosan-HA composite separately with divalent cations of strontium, magnesium and zinc to obtain composite materials with good bioactivity, apatite forming ability, biodegradability and anti-microbial property all in one material.

The reason for selecting these three metal is because they are among the most effective commonly used elements incorporated into the HA structure to enhance its biological response (Cacciotti et al., 2009; Matos et al., 2010; Turki et al., 2013; Webster et al., 2004; Akemi et al., 2012; Kumar, 2012). The doped metals were expected to enhance the dissolution of the chitosan-HA composite hence improving the bioactivity of the materials in addition to the biodegradability biocompatibility and osteoconductivity provided by the HA-composite.

Another main issue associated with the fabrication of chitosan-HA composites is to identify an easier and cost effective method of producing the composites in large quantities. Several methods have been used in the synthesis and preparation of chitosan-HA composites. *Ex situ* and *in situ* method of fabrication have been used to produce these composites Zhang et al., 2005; Nikopour et al., 2012; Mohamed et al., 2014; Yamaguchi et al., 2003) The *ex situ* fabrication methods have the problem of producing a heterogeneous mixture of chitosan and HA. To overcome this issue, *in situ* chemical precipitation methods were identified as some of the easiest and inexpensive techniques of producing a better homogeneous chitosan-HA composite materials in sufficient quantities. Some of these methods used include, *in situ* co-precipitations method (Jin et al., 2008; Nikpour et al., 2012; Rogina et al., 2013), hydrothermal method, biomimetic method (He et al., 2007), facile synthetic and other preparative methods (Mututuvari et al., 2013). In this thesis, a modified *in situ* co-precipitation method was adopted in the synthesis of the composites. This is because co-precipitation method has the advantage of forming extremely supersaturated solution and, hence a very fast