UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT AND EVALUATION OF NOVEL ALGINATE/COCKLE SHELL POWDER NANO-BIOCOMPOSITE POROUS 3D SCAFFOLD FOR BONE REPAIR

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B. HEMABARATHY BHARATHAM

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fufilment of the Requirements for the Degree of Doctor of Philosophy

November 2013

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DEDICATION

This thesis is dedicated with love and appreciation to my beloved;

Father and Mother,
R. Bharatham and S. Mahananda

Husband,
Enoch Kumar Perimal

My brothers, sisters and parent-in laws

For their unconditional love, support, encouragement and trust

For My Son,

Suhail Vihen Enoch Kumar

And

My little nieces and nephew

For the constant reminder of life's simple pleasures beyond academics

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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B. HEMABARATHY BHARATHAM

November 2013

Chairman : Professor Md Zuki Abu Bakar @ Zakaria, PhD

Faculty : Veterinary Medicine

Shells are comparable to bones of vertebrates due to the similarities in mechanical properties and strength. The cockle shell material may act as an anolog of calcium carbonate in an *in vivo* condition that makes it a potential bone grafting material. The present study involves the development and evaluation of a novel three-dimensional alginate/nano cockle shell powder biocomposite bone scaffold prepared through lyophilization and divalent cation cross-linking methods. Element analysis revealed that the shell material consisted of 96% of calcium carbonate with no traces of toxic elements while physiochemical analysis revealed a predominantly aragonite form of calcium carbonate polymorph. The cockle shell powder was converted to nano particles using a biomineralization catalyst through a simple chemical method and was used as a representative of the inorganic phase while sodium alginic acid (alginate) was used as the organic phase in the development of the nano-biocomposite scaffold. The scaffold mixture was prepared in varying composition ratios, characterized and evaluated through various characterization studies. Scanning electron microscopy (SEM) analysis revealed the micro architecture of the

scaffolds with pore size ranging from 10 – 336 μm diameters. The porosity of the scaffolds was found to be above 60%. Mechanical properties of the tested scaffolds showed the composition ratio of 40% alginate and 60% nano cockle powder (Alg:nCP=40:60) possesed favorable mechanical properties ranging between the spongy bone structures compressive strength. Swelling ratio of the scaffolds showed an average of 30% medium uptake ability with 20 – 30% changes in diameter. Enzymatic degradation test revealed an increase in structural stability proportional to the amount of nano cockle shell powder within the composition while pH changes observed during degradation studies revealed a neutralizing effect of nano cockle shell powder towards the potential acidification of the solution during alginate degradation. The physiochemical properties of the materials and the subsequent chemical interactions evaluated revealed the phase purity of the materials as well as the scaffolds ionic interaction characteristics contributing to an increase in thermal stability. *In-vitro* studies conducted on MG63 human osteoblast-like cells revealed good biocompatibility and absences of cytotoxic effect of the scaffolds with higher cell viability noted in scaffolds of 40:60 ratio and was used for further *in-vitro* evaluation. Cell growth and adherence towards the scaffold materials were evaluated for a period of 48 hours, 7 and 14 days using SEM, Element Detection System (EDS) and histological evaluation. The results showed good attachment and spreading properties of the cells within 48 hours and were found to have grown into large cell clusters by Day 7 with distinctive presence of calcium nodules that was verified using EDS analysis on the nano-biocomposite scaffolds. At Day 14, a completely mineralized scaffold structure was observed in the nano-biocomposite scaffolds supported by findings from EDS analysis that showed the presence of phosphate and calcium as well as histological observations showing presence of osteoid like tissues.

In-vivo analysis of the scaffold implanted in a 5 mm osseous defect at the proximal part of the left tibia bone of New Zealand White rabbits revealed evidence of better healing quality of the nano-biocomposite scaffolds compared to control scaffolds as well as empty unfilled defects that were created simultaneously on the right proximal tibia bone of the animals. The quality of healing assessed after seven weeks post implantation through histomorphometric evaluations at three different depths of the defects revealed a significantly better healing in the nano-biocomposite defect site at all three sections compared to the empty defect site as well as with the lower section of the control scaffold defect site. Comparatively, the regeneration of bone tissues were found to occur in a systematic coordinated way with larger areas of matured bone tissues observed in the presences of the nano-biocomposite scaffolds. The remaining void spaces within the defect sites with implants were found to be significantly lesser compared to those of the empty defects while the amount of remaining nano-bicomposite scaffold material was found to be significantly higher compared to the control scaffolds at all three regions evaluated. Statistical analysis for all data's were done using One-way Analysis of Variance (ANOVA) followed by the post-hoc Tukey's test, unless otherwise stated, where $p<0.05$ was accepted as significant. As a conclusion, the developed nano-biocomposite scaffold using alginate and nano cockle shell powder was found to show promising results to be used in the field of bone tissue engineering. The scaffolds showed good porous architectures that enhance its osteoconductive properties by facilitating better and faster bone regeneration in addition to being completely biocompatible as well as a cost effective alternative for bone grafting in the near future

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Key words: Alginate, nano cockle shell powder, porous scaffold, bone tissue engineering, osteoconductive, MG63 osteoblast cells

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan ijazah Doktor Falsafah

FABRIKASI DAN EVALUASI PERANCAH TULANG 3D BERONGGA ALGINATE/SERBUK CENGKERANG NANO BIOKOMPOSIT

Oleh

B. HEMABARATHY BHARATHAM

November 2013

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Kajian ini merangkumi fabrikasi dan evaluasi perancah tulang nano biokomposit tiga dimensi yang disediakan mengunakan komposisi novel serbuk cengkerang kerang fasa nano dan alginat menerusi kaedah 'lyophilization' dan kaedah silang kation divalen. Sifat mekanikal dan kekuatan cengkerang yang didapati setanding tulang speies vertebra di samping bersifat analog kepada kalsium karbonat dalam keadaan *in-vivo* memberikan kelebihan kepada pengunaannya sebagai bahan asas penganti tulang. Analsis unsur mendedahkan bahawa komposisi serbuk cengkerang kerang mengandungi 96% kalsium karbonat tanpa kehadiran unsur-unsur toksik yang lain. Analisis fisiokimia menunjukkan polimorf kalsium karbonat yang hadir adalah berbentuk aragonit. Pembentukan fasa nano serbuk cengkerang dimangkinkan dengan pengunaan 'biomineralization' dan kaedah kimia yang mudah dan digunakan sebagai komponen inorganik dan alginat sebagai komponen organik dalam pembangunan perancah tulang ini. Perancah tulang nano biokomposit telah disediakan dalam komposisi yang berbeza dan dinilai melalui pelbagai kajian pencirian. Struktur mikroskopi perancah yang didedahkan oleh mengimbas analisis

mikroskopi electron (SEM) menunjukkan diameter saiz rongga dalam lingkungan 10-336 μm diameter dengan peratusan keliangan melebihi 60%. Kajian kekuatan mekanikal menunjukkan perancah dengan komposis 40% alginate dan 60% serbuk nano cengkerang (Alg:nCP=40:60) memiliki sifat mekanikal yang baik didalam lingkungan kekuatan mampatan tulang lembut. Kadar keresapan cecair kedalam perancah didapati berpurata 30% manakala kadar perubahan diameternya didapati dalam lingkungan 20-30% saiz asal. Ujian degradasi enzim menunjukkan peningkatan kestabilan struktur perancah dengan penambahan serbuk cengkerang kedalam komposisi. Perubahan pH yang diperhatikan selaras dengan ujian degradasi menunjukkan kesan peneutralan serbuk cengkerang ke arah peningkatan pengasidan akibat degradasi alginat. Sifat fisiokimia perancah yang dianalisis menunjukkan ketulenan fasa dan ciri-ciri interaksi ionik bahan komposisi yang menyumbangkan kepada peningkatan kestabilan terma. Kajian *in-vitro* mengunakan sel osteoblast manusia MG63 menunjukkan ciri keserasian dan ketidakhadiran kesan toksisiti bahan komposisi perancah terhadap sel serta kelebihan komposisi perancah 40:60 dalam menggalakkan kadar petumbuhan sel justeru dipilih sebagai komposisi perancah nano biokomposit dalam kajian-kajian seterusnya. Kajian pertumbuhan sel terhadap perancah dalam tempoh 48 jam, 7 dan 14 hari mengunakan penilaian SEM, EDS dan histologi menunjukkan pelekatan dan pertumbuhan sel yang baik dalam masa 48 jam dan kehadiran kelompok bebola sel besar dalam masa 7 hari serta pembentukkan nodul-nodul kalsium seperti yang disahkan menggunakan analisis EDS pada perancah nano biokomposit. Kadar ‘mineralization’ yang sepenuhnya dapat diperhatikan pada hari ke-14 pada perancah nano biokomposit yang menunjukkan kehadiran fosfat dan kalsium serta tisu osteoid. Kajian *in-vivo* perancah dilakukan menggunakan model arnab (New Zealand White) dengan

kecederaan tulang berbentuk lubang berdiameter 5 mm di wujudkan pada bahagian proksimal kedua-dua tulang tibia arnab di mana tulang kiri diimplan dengan perancah tulang (nano biokomposit atau kawalan) manakala bahagian tulang tibia kanan di biarkan tanpa implan. Kualiti penyembuhan tulang yang dikaji selepas tujuh minggu mengunakan kaedah histologi dan histomorphometric menunjukkan penyembuhan lubang perancah nano biokomposit lebih signifikan berbanding penyembuhan lubang yang tidak dipenuhi pada kesemua tiga bahagian kedalaman yang dikaji serta pada bahagian paling dalam perancah kawalan. Penjanaan semula tisu tulang didapati berlaku secara sistematik dengan kadar kematangan tisu tulang yang lebih tinggi selaras dengan kehadiran perancah nano biokomposit. Ruang yang tidak dipenuhi tisu juga didapati kurang secara signifikan pada lubang yang diimplan perancah berbanding lubang yang tidak dipenuhi manakala jumlah bahan perancah yang masih tertingal didapati tinggi secara signifikan dengan pengunaan perancah nano biokomposit berbanding perancah kawalan. Kesemua analisis statistik telah dilakukan mengunakan Analisis Variance (ANOVA) dan ujian post-hoc Tukey, kecuali dinyatakan sebaliknya dan nilai $p<0.05$ diterima sebagai perbezaan ketara. Kajian ini menunjukkan perancah tulang nano biokomposit yang telah dibangunkan mempunyai ciri-ciri mikroskopik dan liang rongga yang bersesuian untuk memudahkan pertumbuhan tulang serta menunjukkan ciri-ciri keserasian 'biocompatibility' dan 'osteoconductivity' yang mendorong pertumbuhan tulang pada kadar yang lebih cepat. Perancah ini dapati sesuai untuk digunakan dalam bidang kejuruteraan tisu tulang dan menunjukkan potensi yang tinggi dalam menjadi bahan penganti tulang alternatif yang berkos rendah pada masa yang akan datang

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Kata kunci: Alginat, serbuk cengkerang, perancah berongga, kejuruteraan tisu tulang, osteokonduktif, sel osteoblast MG63,

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I certify that a Thesis Examination Committee has met on 14th November 2013 to conduct the final examination of B.Hemabarathy Bharatham on her thesis entitled "Development and Evaluation of Novel Alginate/Cockle Shell Powder Nano-Biocomposite Porous 3D Scaffold for Bone Repair" in accordance with the Universities and University Colleges Act 1971 and the constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The committee recommends that the student be awarded the Doctor of Philosophy degree.

Members of the Thesis Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Date :

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

_____

B.HEMABARATHTY BHARATHAM

Date: 14 November 2013

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LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
AO	Acridine orange
ASTM	American Society for Testing Material
ATCC	American type culture collection
BMP	Bone morphogenic protein
BMU	Basic multicellular unit
$CaCO_3$	Calcium carbonate
DBM	Demineralized bone matrix
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDS	Element detection system/ Energy-dispersive X-ray spectroscopy
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor-2
FTIR	Fourier transform infrared spectrophotometer
G-blocks	α-L guluronic acid
HA	Hydroxyapatite
ICP-MS	Inductive coupled plasma mass spectrometry
IGF	Insulin like growth factor
IL	Interleukins
JCPDS	Joint committee of powder diffraction society
M- blocks	β-D mannuronic acid

MCSF	Macrophage colony stimulating factor
MTT	3-dimethylthiazo-2, 5-diphynyltetrazolium bromide
PBS	Phosphate buffer solution
PGA	Poly (glycolic acid)
PI	Propidium iodide
PLA	Poly (L-lactic acid)
PLGA	Poly (D,L-lactic acid co-glycolic acid)
pNPP	p-nitrophenyl phosphate
RANK	Receptor activator of nuclear factor kappa β ligand
SEM	Scanning electron microscopy
TCP	Tricalcium phosphate
TGA	Thermogravimetric analysis
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor- α
XRD	X-ray diffractometer

CHAPTER 1

GENERAL INTRODUCTION

The dreams of restoring a damaged body have been in existence since the start of humankind with early history manifesting them as myths and magic. New understanding of the natural world, disease, trauma and the introduction of scientific methods enabled production of artificial prosthetic materials to restore the lost functions of organs and tissues. With the unfolding of the 16th century, the concept of replacing one tissue with another was developed. This has laid the foundation for the emergence of the field of tissue engineering which formally begun in 1987 (Vacanti and Vacanti, 2007). The science of designing and fabricating new tissues or materials for impairment repairs has since been widely studied and is constantly expanding. The bone possessing the highest regeneration potentials provides a classic example of a clear principle of a tissue engineering model (Fisher and Reddi, 2003).

The human bone represents one of the most important organs of the human body. These rigid organs play an essential role in providing the needed support, protection and movement. Bones are one of the very few human organs that possess the potentials of regenerating and the only organ capable of remodeling. The normal physiological condition of the bone involves a constant formation and resorption phase that allows 5-15% of remodeling of the total bone mass in a year (Perez-Sanchez *et al.*, 2010). These unique features of the bones are well manipulated in the field of tissue engineering in a constant search for an ideal bone replacement material.

Bone replacement or transplantation involves the grafting of a new bone or a suitable replacement material between the spaces of a fractured bone or a defected bone in order to aid the healing process. The process is often undertaken in situations in which the fractures are extremely complex with large areas of segmental defect or bones that have failed to heal properly. In such cases, bone grafts are used to help the fusion of the damaged bones, correct deformities as well as to provide the needed structural support.

Bone grafting

Over a century, the process of bone grafting has been utilized by orthopedic surgeons due to the constant need for bone replacement. Bone replacement becomes an inevitable part of orthopedic applications due to injuries caused by traumatic processes or physiological degenerative processes that leads to bone loss that compromises the quality of an individual's life. In the United States alone, it is estimated over 500 000 bone-grafting procedures are performed annually (Boden, 2003). This scenario further adds to the improvement of the existing methods of bone grafting. The longing for new and improved bone grafts has increased the innovativeness using tissue engineering paradigm to develop effective products during the last decade (Ilan and Ladd, 2002)

In the field of bone grafting, the golden standard procedure of transplantation is autologus bone grafting. Though this has been practiced over decades, the requirement of a second surgical site with limited quantity of donor tissue as well as possible risk of donor site morbidity and an increased operative time and cost poses

the possible constraints in conducting this procedure. Optional to these, includes procedures such as allograft which involves the transplantation of tissues from donors as well as xenograft that involves transplantation of animal originated tissues. With each of these procedures, potential risk of pathogen transmission, immunological responses and infections constantly is an inherent problem (Lee and Shin, 2007).

Bone grafting requirements

The success of a bone grafting procedure involves the principals of osteoconduction, osteoinduction and osteogenesis in addition to providing the needed mechanical stability, support and strength. The guided reparative growth of the natural bone (osteoconduction) and increased activation of mesenchymal cells to form active osteoblast cells (osteoinduction) are characteristics of many grafting materials with osteoconduction properties being predominant. To date only autografts fulfill all principal requirements of a successful bone graft as well as being the only true osteogenic material capable of inducing direct bone formation (Ilan and Ladd, 2002). Autografts offer no immunological rejection while providing considerable ostoconductive, osteoinductive and osteogenic properties (Samartzis *et al.*, 2005). However, given the considerable amount of limitations in using autografts as bone replacement materials, graft substitutes represent a more interesting and appealing option for bone grafting procedures (Ilan and Ladd, 2002). Substitutes for autografting include allografts, mineral composites, injectable cements, bioactive glass, polymers, proteins and growth factors. A large number of graft substitutes currently exist from the various classes mentioned above with their use and

availability expending exponentially each year. Despite the existence of these products and a market filled with unlimited options, the ideal implant material that could mimic an actual bone regenerative capacity is yet to be invented.

The expectation of a graft substitute is highly dependent on the nature of the fracture or defect of the bone. This determines the use of the graft whether as a simple void filler or as a larger gap filler that acts as a scaffolding material to facilitate new bone formation. In both cases, the graft material acts as a structural support and strength provider (Ilan and Ladd, 2002). To date, the choice of graft substitute marketed fulfills these criteria and one or more of the key principals of bone healing (osteoconduction, osteoinduction and osteogenesis) but not all. At the very least a grafting material designed should be osteoconductive in nature to be used as simple void fillers facilitating the formation of new bone cells. With the incorporation of growth factors such as Bone Morphogenic Proteins' (BMPs) that promotes cell growth, an osteoinductive nature could be conferred to a grafting material to promote an even faster rate of healing. This desired stimulation for bone growth though has been claimed to be present in human allografts, are still lacking in scientific evident as it has not been specifically identified despite its marketing claims shown through many successful animal based studies (Ilan and Ladd, 2002). The lack of comparative human studies that could clearly demonstrate the benefit of one grafting materials with another has created a void in the choice of bone graft substitutes that is considered as an ideal material. The constant emergence of newly innovated or improved grafting materials keeps the field of bone tissue engineering an exciting avenue for future studies in order to fulfill these empty voids in producing a grafting material that fulfills the principals of a successful bone substitute material.

Biomaterials and Bone Tissue Engineering

In line with the trend, the use of biomaterials in graft substitute materials has recently emerged as an inspiring development in this field, with the third generation biomaterials being developed lately. This materials could be designed to provide cells for osteogenesis with osteoconduction properties as well as to contain osteoinduction signals in which these factors can be used separately or in combination (Scheller *et al.*, 2009; Torroni, 2009). The biomaterials used in the field of bone tissue engineering are often produced in the form of a scaffolds that acts as a highly specialized bone grafting material that will be able to provide a three dimensional model mimicking the extracellular matrix for the adhesion, cell proliferation and differentiation in addition to conferring the needed structural support for the protection of early stages of tissue healing (Freed *et al.*, 1994). The biomaterial based graft substitutes are also being designed to achieve the needed bioactivity with capabilities of provoking a specific cellular response at molecular level in addition to be able to reabsorb with time (Hench and Polak, 2002). These materials should not illicit an adverse reaction when placed into the biological environment.

These current concepts in bone tissue engineering forms the basis for the current research work in order to develop a new bone scaffold using naturally occurring biomaterials. In this study, shell powder were used as a source of calcium carbonate aragonite polymorph in combination with alginic acid (alginate), a naturally occurring polymer, in order to produce a three dimensional scaffold for possible bone grafting applications.

Hypothesis of the Study

The nano-biocomposite bone scaffold fabricated is able to mimic the natural structure of bones and is able to produce sufficient osteoconductive response in order to promote better bone healing as well as to display the desired characteristics of an ideal bone grafting material.

Objectives of the Study

The objectives of the study were to:

i. analyze the composition and chemical properties of cockle shell and other selected molluscan shell powder as a potential biomaterial for bone tissue engineering.

ii. develop and characterize alginate/cockle shell powder novel nano-biocomposite porous three dimensional (3D) scaffold.

iii. conduct an *in-vitro* evaluation of the nano-biocomposite scaffold for biocompatibility and bone tissue regeneration potentials.

iv. investigate the osteogenic ability and tissue compatibility of the prepared scaffold through *in-vivo* evaluation using an appropriate animal model.

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