
**UNIVERSITI PUTRA MALAYSIA**

# *In Vitro EVALUATION OF COCKLE SHELL-BASED CALCIUM CARBONATE ARAGONITE POLYMORPH NANOPARTICLE WITH SURFACE FUNCTIONALIZATION FOR DRUG DELIVERY APPLICATIONS*

**SYAIRAH LIYANA BINTI MOHD ABD GHAFAR**

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**By**

**SYAIRAH LIYANA BINTI MOHD ABD GHAFAR**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science**

**December 2016**

Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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**Chairman : Professor Md Zuki Abu Bakar @ Zakaria, PhD**
**Institute : Bioscience**

Drug delivery is a current biomedical application employing nano-sized particles. In line with current global interest, employing nature-based materials to construct delivery carriers has been highly preferable due to their environmental friendly, availability, low cost, and good natural mineral purity. Cockle (*Anadara granosa*) shells was reported to contain comparable mineral compositions to vertebrates bone with high calcium carbon and no evident presence of heavy metal elements with good quality and pure calcium carbonate aragonite crystals. In order to meet stringent qualities of drug carrier for drug delivery applications, an improved synthesis method incorporated with surface functionalization was developed to produce nanoparticles with high homogeneity in size and shape. The study aimed at evaluating the physicochemical characteristics of surface functionalized cockle shell-based calcium carbonate aragonite nanoparticle and its potentials as delivery agent. Cockle shell micron-sized powder was converted into nano-sized particles through a mechanical stirring process in the presence of dodecyl dimethyl betaine (BS-12). The effect of BS-12 surfactant on the surface property of cockle shell-based calcium carbonate aragonite nanoparticles was analyzed through pH evaluation, Fourier Transform Infrared (FTIR) and X-ray diffraction (XRD) analyzes. Transmission Electron Microscopy (TEM) and Field Emission Scanning Electron Microscopy (FESEM) demonstrated agglomeration of nanoparticles after the addition of surfactant. However, with calcium ion adsorption onto the surface of cockle shell-based calcium carbonate aragonite, the dispersion of the nanoparticles has improved as shown by the increase in zeta potential. Purification technique further enhanced the overall distribution of nanoparticles towards more refined size range of less than 100 nanometers, which is favorable for drug delivery applications. The purity of aragonite phase and chemical functionality were verified by FTIR and X-ray diffraction (XRD) analyzes. *In vitro* biological response on human fetal osteoblast (hFOB 1.19) cell line demonstrated that surface functionalization could decrease cytotoxicity. Surface functionalized cockle shell-based calcium carbonate aragonite nanocarrier showed

better capacity to load drug molecules and was able to sustain incorporation of some drug molecules up to three days *in vitro*. Both the cockle shell-based nanocarrier samples were sensitive to pH changes as they released more drug compounds in the acidic environment of pH 6.4 than pH 7.4. This new delivery agent from cockle shells may provide an alternative source for calcium carbonate aragonite polymorph nanoparticles as an efficient drug carrier for therapeutic applications.

**Keywords:** cockle shell, calcium carbonate, surface functionalization, drug delivery

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

# PENILAIAN *In Vitro* PARTIKEL NANO KALSIUM KARBONAT POLIMORF ARAGONIT DARIPADA KULIT KERANG DENGAN MODIFIKASI PERMUKAAN UNTUK APLIKASI PEMBAWA UBAT


Oleh

**SYAIRAH LIYANA BINTI MOHD ABD GHAFAR**


**Disember 2016**


**Pengerusi : Profesor Md Zuki Abu Bakar @ Zakaria, PhD**
**Institut : Biosains**



Pembawa ubat adalah aplikasi bioperubatan terbaharu yang menggunakan zarah bersaiz nano. Selaras dengan kepentingan global, penggunaan bahan daripada alam semula jadi adalah lebih baik oleh kerana faktor mesra alam, ketersediaan sumber, kos yang rendah, dan memiliki komponen mineral semula jadi yang murni. Kulit kerang (*Anadara granosa*) memiliki komposisi mineral kalsium karbon yang tinggi yang hampir menyamai tulang vertebra, tidak mengandungi elemen logam berat serta mempunyai kandungan kristal kalsium karbonat aragonit yang berkualiti baik dan tulen. Suatu kaedah penghasilan partikel nano yang dipertambahbaik telah dicipta untuk menghasilkan partikel nano yang mempunyai saiz dan bentuk yang seragam untuk tujuan aplikasi pembawa ubat. Kajian ini dilakukan untuk menilai sifat fizikal, kimia serta potensi partikel nano kalsium karbonat kulit kerang yang dimodifikasi sebagai pembawa ubat. Kulit kering yang telah diproses menjadi serbuk halus bersaiz mikron akan dihasilkan menjadi partikel nano melalui suatu proses mekanikal dengan menggunakan bahan surfaktan iaitu *dodecyl dimethyl betaine* (BS-12). Kesan penggunaan bahan surfaktan terhadap sifat permukaan partikel nano kulit kerang telah dianalisis melalui pemeriksaan pH, analisis FTIR, dan XRD. Pemeriksaan mikroskop elektron TEM dan FESEM mendapati bahawa penggumpalan partikel nano terjadi setelah penambahan bahan surfaktan. Namun begitu, proses modifikasi permukaan telah menyebabkan berlakunya adsorpsi ion kalsium pada permukaan partikel nano kulit kerang sehingga ia dapat memperbaiki penyebaran partikel yang telah diindikasikan oleh peningkatan nilai potensi zeta. Teknik penulenan yang dianjurkan juga telah berjaya menghasilkan partikel nano yang mempunyai distribusi saiz yang sesuai untuk aplikasi pembawa ubat yaitu kurang daripada 100 nanometer. Pemeriksaan FTIR dan XRD telah mengesahkan ketulenan kristal aragonit dan fungsi kimianya. Hasil penilaian *in vitro* terhadap sel tulang fetus manusia (hFOB 1.19) mendapati bahawa proses modifikasi yang dilakukan telah berjaya mengurangi ketoksikan bahan terhadap sel. Partikel nano kalsium karbonat kulit kerang yang dimodifikasi juga telah menunjukkan kebolehan membawa ubat yang lebih baik serta ia berjaya mempertahankan kandungan molekul ubat sehingga tiga hari secara *in vitro*.

Kedua jenis partikel nano kulit kerang dengan atau tanpa modifikasi bersifat sensitif terhadap perubahan pH di mana, kedua-duanya melepaskan komponen ubat lebih cepat dalam persekitaran berasid yaitu pada pH 6.4 berbanding pH7.4. Partikel nano kalsium karbonat aragonit berasaskan kulit kerang ini dapat menjadi suatu sumber alternatif sebagai pembawa ubat yang efisien untuk pelbagai aplikasi terapi.

**Kata kunci**: kulit kerang, kalsium karbonat, mofikasi permukaan, pembawa ubat

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I certify that a Thesis Examination Committee has met on 8 December 2016 to conduct the final examination of Syairah Liyana binti Mohd Abd Ghafar on her thesis entitled "*In Vitro* Evaluation of Cockle Shell-Based Calcium Carbonate Aragonite Polymorph Nanoparticle with Surface Functionalization for Drug Delivery Applications" 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 Master of Science.

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Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

**Rasedee @ Mat bin Abdullah, PhD**
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

**Ahmad Hafiz bin Zulkifly, PhD**
Professor
International Islamic University Malaysia
Malaysia
(External Examiner)

**NOR AINI AB. SHUKOR, PhD**
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 22 March 2017

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

**Md. Zuki bin Abu Bakar @ Zakaria, PhD**
Deputy Director of Institute of Bioscience
Professor of Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

**Mohd Zobir Hussein, PhD**
Professor
Institute of Advanced Technology
Universiti Putra Malaysia
(Member)

**Yaya Rukayadi, PhD**
Associate Professor
Institute of Bioscience
Universiti Putra Malaysia
(Member)

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| Signature: | |
| Name of Member of Supervisory Committee: | Professor Dr. Mohd Zobir Hussein |
| Signature: | |
| Name of Member of Supervisory Committee: | Associate Professor Dr. Yaya Rukayadi |

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of variance |
| ATCC | American type culture collection |
| BS-12 | Dodecyl dimethyl betaine |
| $CaCl_2.2H_2O$ | Calcium chloride dihydrate |
| $CaCO_3$ | Calcium carbonate |
| CSD | surface functionalized cockle shell-based calcium carbonate aragonite nanoparticle loaded drug complex |
| CXSD | cockle shell-based calcium carbonate aragonite without surface modification nanoparticle loaded drug complex |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethyl sulfoxide |
| FBS | Fetal bovine serum |
| FESEM | Field emission scanning electron microscopy |
| FTIR | Fourier transform infrared spectrophotometer |
| JCPDS | Joint committee of powder diffraction society |
| mL | milliliters |
| MTT | 3-dimethylthiazo-2, 5-diphynyltetrazolium bromide |
| nm | Nanometers |
| PBS | Phosphate buffered solution |
| ppm | parts per million |
| SD | Standard deviation |
| TEM | Transmission electron microscopy |
| µm | Micrometers |
| XRD | X-ray diffractometer |

# CHAPTER 1

# GENERAL INTRODUCTION

Nanotechnology is no longer a new branch of studies in the science. Generally, nanotechnology does not focus on any specific area of study but it is actually a multidisciplinary field of sciences that covers and greatly influences many areas in our lives. It has successfully contributed a myriad of advancements in the development of various industries including in construction, aerospace defense, engineering, medicine, pharmaceuticals, cosmetics, sports, electronics, automotive, and chemical industries. Some products and applications pertaining to nanotechnology, such as cosmetics and pharmaceutical products, are in fact already available in the market (Mishra *et al*., 2010; Parveen *et al*., 2012; Silpa *et al.*, 2012).

There are many definitions of nanotechnology that was made to elaborate the term precisely. For example, the National Nanotechnology Initiative (NNI) by the National Science and Technology Council of the United States government defines nanotechnology as "an encompassing term for nanoscale science, engineering, and technology that involves imaging, measuring, modeling, and manipulating matter via the understanding and control of matter at dimensions between approximately 1 and 100 nanometers, where unique phenomena enable novel applications" (NSTC, 2004). The Malaysian Ministry of Science, Technology, and Innovation, via its National Nanotechnology Directorate Division (NND) in July 2010, has come out with a simpler definition of nanotechnology that is "the creation and utilization of material, devices and systems through the manipulation of matter at scales of less than 100 nanometers" (MOSTI, 2010). Although those definitions come from different sources, their essential objective is directed towards exploitation of nano-sized matter for advanced innovation in various applications.

Implementation of nanotechnology that basically focuses on the medical science field is known as nanomedicine. The term was defined as "monitoring, repair, construction and control of human biological systems at the molecular level using engineered nanodevices and nanostructures" (Robert, 1999). Some applications of nanotechnology in medicine are promising and have indeed offered many advantages in various medical areas such as drug delivery to specific sites, gene delivery in gene targeted therapy, molecular imaging for diagnostic and therapeutic intervention, cardiac therapy, dental care as well as orthopedic applications (Sahoo *et al*., 2007; Robert, 2009; Mishra *et al*., 2010). In fact, numerous applicable nanotechnology-based biomedical devices are already available in the current market and there are also some products undergoing clinical trials (Sahoo *et al*., 2007; Parveen *et al.*, 2012).

Drug delivery is one of the current biomedical advances employing nano-sized particles for either diagnostic or therapeutic purposes based on nanomedicine concept. Ideally, drug delivery system is for the transport drug molecules to particular sites without affecting normal tissues or other internal organs (Zhi-Ping *et al*., 2006; Amir

and Peter, 2009; Mishra *et al*., 2010; Parveen *et al*., 2012). On the other hand, there are some limitations encountered by the conventional drug delivery system. For instance, direct delivery by conventional system might experience problem relating to enzymatic degradation of drugs compounds before they could safely arrive at the specified targets (Zhi-Ping *et al*., 2006; Rajesh and James, 2009; Amir and Peter, 2009). Besides, some therapeutically active molecules could also experience difficulty in reaching arduous sites of action, attaining optimal therapeutic level while targeting desired location, and controlling drug release (Zhi-Ping *et al*., 2006; Sahoo *et al*., 2007; Amir and Peter, 2009; Rajesh and James, 2009; Parveen *et al.*, 2012).

To date, extensive efforts was devoted in the search for an appropriate key to overcome some of the problems of drug delivery via research and development programs (Ueno *et al.*, 2005; Rajesh and James, 2009; Jae-Hyung *et al.*, 2010; Parveen *et al.*, 2012; Anil *et al*., 2012; Sang-Kyoon *et al*., 2012; Yulia *et al.*, 2013; Shafiu *et al.*, 2013). The demanding exploration for delivery agents remains ongoing covering many aspects of research including on types, physical, and chemical properties of materials as well as surface characterization of delivery carriers. In fact, many materials were intensively studied in the construct of efficient and effective carriers including inorganic materials, carbon tube, gold, silver, and polymer-based materials (Ueno *et al*., 2005; Mahendra *et al.*, 2009; Chen *et al*., 2011; Satya *et al.*, 2011; Forrest *et al.*, 2011; Anil *et al*., 2012; Folusho and Richard, 2012; Shafiu *et al.*, 2013). Although there are variety choices of materials, calcium carbonate represents one of the inorganic materials of choice used to devise the delivery vehicles in drug delivery system (Ueno *et al.*, 2005; Dong *et al.*, 2012; Sang-kyoon *et al.*, 2012; Shafiu *et al.*, 2013; Yulia *et al.*, 2013; Ping *et al.*, 2013).

A large number of studies was also conducted to investigate some properties of the calcium carbonate material (Jiaguo *et al.*, 2004; Zeshan and Yulin, 2004; Ueno *et al.*, 2005; Guowei *et* al., 2009; Meng-Chun and Clifford, 2010; Hoang-Vinh *et al.*, 2010; Kwang-Min and Kazuyuki, 2011; Sargheini *et al.*, 2012; Dong *et al.*, 2012; Nobuyoshi *et al.*, 2013). Calcium carbonate consists of three kind polymorphs which are calcite, aragonite, and vaterite. Each of these polymorphisms possesses different properties that define their special characteristics. Currently, the physical and chemical properties of calcium carbonate polymorphisms have been established (Kamiya *et al.*, 1977; Sohnel and Mullin, 1982; Laifeng *et al.*, 1999; Mitsutaka, 2002; Xiang *et al.*, 2004; Chengyu *et al.*, 2006; Rizzuti and Lionelli, 2008; Zeshan *et al.*, 2009; Hongxia *et al.*, 2011; Sargheini *et al.*, 2012; Nobuyoshi *et al.*, 2013).

Aragonite polymorph of calcium carbonate has high potential as a good biomedical material that could be integrated, resolved, and replaced by bones owing to its some unique characteristics (Stupp and Braun, 1997; Chengyu *et al*., 2006; Islam *et al.*, 2011). Although aragonite is thermodynamically less stable than calcite at ambient temperature and pressure, but it is denser than calcite, sensitive towards temperature changes, possesses beneficial properties such as high mechanical strength, biocompatibility, and biodegradability (Stupp and Braun, 1997; Zuki *et al.*, 2004; Chengyu *et al*., 2006; Awang-Hazmi *et al.*, 2007; Islam *et al*., 2011; Shafiu *et al*., 2013). For those reasons, this material has become a current focus in many fields of

biomedical research including drug delivery and bone tissue engineering (Zuki *et al.*, 2011; Shafiu *et al.*, 2013).

Surface functionalization can improve the interaction between carriers and specific cell membranes through manipulation on the surface charge of particles and thus help the carrier binding to targeted locations (Zhi-Ping *et al*., 2006; Amir and Peter, 2009; Rajesh and James, 2009). In fact, the interaction among particles as well as the interaction between nanoparticles and drug compounds in suspension can be enhanced through modifications of surface property of the particles to affect their surface energy and electrical potential hence improving the dispersion as well as distribution of nanoparticles in the body (Huang *et al.*, 1990; Xu *et al*., 2005; Chengyu *et al.*, 2007; Hoang-vinh *et al.*, 2010; Ping *et al.*, 2013).

From the fabrication of micron-sized calcium carbonate particles in the early days up to the synthesis of desirable nano-sized particles today, many studies have used the bottom-up approach via the precipitation process, either through carbonation or solution route for the production of inorganic calcium carbonate raw material over the past few years (Chengyu *et al.*, 2006; Meng-Chun and Clifford, 2010; Hoang-Vinh *et al.*, 2010; Hongxia *et al.*, 2011; Sargheini *et al.*, 2012; Dong *et al.*, 2012; Yulia *et al*., 2013). Many researchers recently started diverting their interest from synthetically methods to produce calcium carbonate to the utilization of nature-based biogenic materials using the top-down process of calcium carbonate nanoparticle production. In fact, a number of studies have derived calcium carbonate compound from naturally occurring byproduct of cockle shells for many purposes especially in biomedical applications (Zuki *et al.*, 2004; Awang-Hazmi *et al*., 2007; Islam *et al.*, 2011; Islam *et al.*, 2012; Shafiu *et al*., 2013).

The cockle belongs to the *Anadara granosa* species, a type of sea molluscan that could be found in the intertidal sea and mudflat areas in the coastal region of Southeast Asian countries such as Malaysia, Indonesia, and Thailand (Awang-Hazmi *et al*., 2007). In fact, several studies (Zuki *et al*., 2004; Awang-Hazmi *et al*., 2007; Islam *et al.*, 2011; Shafiu *et al.*, 2013; Hemabarathy *et al.*, 2014) have successfully shown that calcium carbonate derived from cockle shells is suitable and safe for biomedical applications. Besides the vast availability and abundance, cockle shells are convenient, economic and environmental-friendly resource of calcium carbonate. Furthermore, calcium carbonate biomaterial derived from naturally occurring cockle shells contains good quality and pure aragonite polymorph of calcium carbonate and therefore, it is a very suitable biomaterial source for application in biomedical and tissue engineering studies (Zuki *et al*., 2004; Awang-Hazmi *et al*., 2007; Islam *et al*., 2011; Zuki *et al.*, 2011; Shafiu *et al.*, 2013).

A fundamental study by Islam *et al.* (2011) has established a basic preparation method to process the cockle shells into micron-sized powder and also developed a novel preparation method using simple mechanical stirring in the presence of Dodecyl dimethyl betaine or commonly known as BS-12. Shafiu *et al.* (2013) has also introduced another preparation method of cockle shell-based calcium carbonate

nanoparticles production using a high pressure homogenizer (HPH) technique via microemulsion route in the presence of polysorbate 80 (Tween 80) surfactant. Even though the technique has shown promising results for drug delivery application, but given considerable limitations such as requirement of complex and expensive equipments with high energy input to operate, the simple stirring method by Islam *et al*. (2011; 2012) is a more appealing and convenient option for facile preparation of calcium carbonate aragonite nanoparticles even for a large-scale production of calcium carbonate aragonite polymorph nanoparticles.

Adjuring to the recent trend in drug delivery system, the present research employed the method introduced by Islam *et al*. (2012) with some improvisation, in the synthesis of calcium carbonate aragonite polymorph nanoparticle. The nanoparticle production method was also designed to incorporate surface functionalization process in order to achieve the required bioactivity and efficiency as a delivery carrier in terms of size, morphology, surface property, drug loading and release while attaining low cytotoxic towards biological systems.

**Problem Statements**

In line with current global interest, employing nature-based materials to construct delivery carriers is highly preferable due to their environmental friendly, availability, low cost, and good natural mineral purity. Cockle (*Anadara granosa*) shells was reported to contain comparable mineral compositions to vertebrates bone with high calcium carbon and no evident presence of heavy metal elements with good quality and pure calcium carbonate aragonite crystals.

The synthesis of calcium carbonate nanoparticles from naturally occurring cockle shells in the presence of dodecyl dimethyl betaine was previously developed, but further research is required to improve the synthesized method for high homogeneity production of nanoparticles in size and shape for drug delivery application.

To the best of our knowledge, there is no published study investigating on the controlled surface of cockle shells-based calcium carbonate aragonite polymorph nanoparticles as present drug carrier. Therefore, the effect of surface functionalization on the cockle shells-based calcium carbonate aragonite polymorph nanoparticles was evaluated.

The investigation of physicochemical characteristics of surface functionalized cockle shell-based calcium carbonate aragonite polymorph nanoparticles is important to assess its potential qualities as a promising delivery vehicle in drug delivery systems.

An efficient drug carrier should have low cytotoxicity with good abilities to load and release drugs. In this study, the cytocompatibility of surface functionalized cockle shells-based calcium carbonate aragonite polymorph nanoparticle and its efficacy to load and release drug were thus investigated.

**Study Hypothesis**

Surface functionalized cockle shell-based calcium carbonate aragonite polymorph nanoparticle has characteristics of an efficient drug nanocarrier in terms of zeta potentials, dispersion, and stability in solution, cytocompatibility, low cytotoxicity, as well as excellent drug loading and release.

**Study Objectives**

**General objective**

This research aims to assess the potential of surface functionalized cockle shell-based calcium carbonate aragonite polymorph nanoparticles for a drug delivery system.

**Specific objectives**

i. To prepare and evaluate the physical and chemical properties of surface functionalized cockle shell-based calcium carbonate aragonite polymorph nanoparticles for drug delivery.

ii. To determine the *in vitro* efficacy of surface functionalized cockle shell-based calcium carbonate aragonite polymorph nanoparticles in terms of cytotoxicity, drug loading and release as a drug nanocarrier system.

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## LIST OF PUBLICATIONS

Syairah L.M.A.G., Mohd, Z.H. and Zuki, A.B.Z. 2017. Synthesis and characterization of cockle shell-based calcium carbonate aragonite polymorph nanoparticles with surface functionalization. *Journal of Nanoparticles*, 2017: 12.

Syairah L.M.A.G., Mohd, Z.H., Yaya, R. and Zuki, A.B.Z. Surface-functionalized cockle shell-based calcium carbonate aragonite polymorph for nanocarrier of drug. *Nanotechnology, Science and Applications*, (Status: In-press).

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