UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT AND IN VITRO BIOEVALUATION OF COCKLE SHELLCALCIUM CARBONATE (ARAGONITE) NANOPARTICLES FOR INTRACELLULAR DRUG DELIVERY

TIJANI ISA

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By

TIJANI ISA

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

October, 2015

DEDICATION

This thesis is sincerely dedicated to the memory of my late parents Sheikh Isa Ladan Yakub and Hafsat Isa Ladan Yakub and my step-mammy Aishat Isa Ladan Yakub (May your souls continue to rest in Jannah). You will forever remain in my dear heart.

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfillment of the requirements for the Degree Master of Science.

DEVELOPMENT AND *IN VITRO* BIOEVALUATION OF COCKLE SHELL-CALCIUM CARBONATE (ARAGONITE) NANOPARTICLES FOR INTRACELLULAR DRUG DELIVERY

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

The use of safe and efficient delivery systems, capable of delivering therapeutic agents to sub-cellular levels are an ultimate goal in enhancing therapeutic effect. It is also a promising strategy in overcoming microbial resistance and the emergence of intracellular bacterial infections. The challenge, however, is that the interaction of nanoparticles with biological systems at the cellular level must be established prior to biomedical applications. In this study, ciprofloxacin conjugated cockle shells-derived calcium carbonate (aragonite) nanoparticle (C-CSCCAN) was developed and characterized for its physicochemical properties and antibacterial activities. Biocompatibilities were evaluated on macrophage cell line (J774.A1) using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 5-Bromo-2′-deoxyuridine (BrdU) assays. The nanoparticles were spherical in shape, with particles sizes ranging from 11.93 to 22.12 nm as determined through a transmission electron microscope (TEM). The highest percentage entrapment efficiency (EE) and loading content (LC) were 99.5% and 5.9%, respectively, with an optimum negative zeta potential. X-ray diffraction (XRD) patterns revealed strong crystallity of the formulations. Fourier transforms infrared (FT-IR) spectra shows evident of interactions exist between the drug and nanoparticles at the molecular level. No burst effect, but a sustained drug release was observed from the formulation. The mean diameter of inhibition zone was 18.6 ± 0.5 mm, which was better than ciprofloxacin alone (11.7 ± 0.9 mm), while the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the formulation were lower than those of free drugs. Study of biocompatability suggested non-toxic effects of the formulations. In conclusion, the results indicated that the ciprofloxacin- nanoparticle conjugate (C-CSCCAN) enhanced susceptibility of *Salmonella* and antibacterial efficacy of the antibiotic, which could potentially improve the clinical efficacy of the drug.

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

PEMBANGUNAN DAN PENILAIAN BIOLOGI SECARA *IN VITRO* KE ATAS KALSIUM KARBONAT (ARAGONITE) NANOPARTIKEL UNTUK PENGHANTARAN DADAH INTRASEL

Oleh

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Penggunaan sistem penghantaran selamat dan cekap, mampu menyampaikan agen terapeutik ke tahap sub-selular merupakan matlamat utama dalam meningkatkan kesan terapeutik. Ia juga merupakan satu strategi yang boleh dipercayai dalam mengatasi rintangan mikrob dan kemunculan jangkitan bakteria intrasel. Cabaran itu, bagaimanapun, adalah bahawa interaksi nanopartikel dengan sistem biologi pada tahap sel perlu diwujudkan sebelum aplikasi bioperubatan. Dalam kajian ini, ciprofloxacin terkonjungsi kalsium karbonat (aragonite) nanopartikel dari cengkerang kerang (C-CSCCAN) telah dibangunkan dan mempunyai ciri-ciri fizikokimia dan aktiviti anti-bakteria. Biocompatibiliti telah dinilai pada baris sel makrofaj (J774.A1) menggunakan 3-(4,5-Dimethylthiazol-2-YL)-2,5-diphenyltetrazolium bromida (MTT) dan 5-Bromo-2'-deoxyuridine (BrdU) asei. Nanopartikel adalah berbentuk bulat, dengan zarah saiz antara 11.93 – 22.12 nm seperti yang ditentukan melalui mikroskop elektron transmisi (TEM). Kecekapan peratusan pemerangkapan (EE) dan kandungan yang dimuatkan (LC) yang tertinggi adalah masing-masing 99.5% dan 5.9%, dengan potensi zeta negatif yang optimum. Pola pembelauan sinar-X (XRD) mendedahkan kristaliti yang kuat daripada formulasi. Spektrum fourier mengubah inframerah (FT-IR) menunjukkan bukti wujud interaksi diantara dadah dan nanopartikel pada peringkat molekul. Tiada kesan pancutan rembesan dadah, tetapi perembesan dadah yang berterusan diperhatikan dari formulasi. Garis pusat min zon perencatan adalah 18.6 ± 0.5 mm, adalah lebih baik daripada ciprofloxacin sahaja (11.7 ± 0.9 mm), manakala kepekatan perencatan minimum (MIC) dan kepekatan bakteria minimum (MBC) formulasi adalah lebih rendah daripada yang dadah sahaja. Kajian biocompatabiliti mencadangkan tiada kesan toksik daripada formulasi. Kesimpulannya, keputusan menunjukkan bahawa ciprofloxacin terkonjugsi kalsium karbonate nanopartikel (C-CSCCAN) meningkatkan kecenderungan *Salmonella* dan keberkesanan anti-bakteria antibiotik, yang berpotensi meningkatkan keberkesanan klinikal dadah.

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I certify that a Thesis Examination Committee has met on 28 October 2015 to conduct the final examination of Tijani Isa on his thesis entitled "Development and *In Vitro* Bioevaluation of Cockle Shell- Calcium Carbonate (Aragonite) Nanoparticles for Intracellular Drug Delivery" 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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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BrdU	5-bromo-2′- deoxyuridine
$CaCO_3$	Calcium Carbonate
Ca^{2+}	Calcium ion
C-CSCCAN	Ciprofloxacin-cockle shells calcium carbonate aragonite nanoparticles
	Colony-forming unit
CFU	Clinical and Laboratory Standards Institute
CLSI	Centimeter
Cm	Carbon dioxide
CO_2	
CSCCAP	Cockle shells calcium carbonate aragonite nanoparticles powder
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EE	Encapsulation efficiency
FBS	Foetal Bovine Serum
FESEM	Field Emission Scanning Electron Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
HPH	High Pressure Homogenizer
H_2O	Water
LC	Loading Capacity
MBC	Minimum Bactericidal Concentration
mg/mL	Milligram per milliter
MHA	Mueller–Hinton Agar
MHB	Mueller–Hinton Broth
MIC	Minimum Inhibitory Concentration
Min	Minute(s)
mL	Milliliter
Mm	Mircometer

Mm	Millimete
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5diphenyltetrazolium bromide
Mv	Millivolts
Nm	Nanometer
PBS	Phosphate Buffer Saline
Rpm	*Revolutions per minute*
R^2	Regression square
SD	Standard deviation
TEM	Transmission Electron Microscopy
µg/mL	Microgram per millilitre
Wf	Weight of free drug
WnP	Weight of nanoparticles
Wt	Total weight of drug fed
W/O	Water in Oil microemulsions
XRD	X-ray Diffractometry
^oC	Degree Celsius

CHAPTER 1

INTRODUCTION

1.1 Background

Facultative intracellular bacterial pathogens are notorious causative agents for a number of severe diseases world-wide. These pathogenic agents have developed a number of inventive mechanisms to replicate and spread inside numerous multi-cellular eukaryotes including the antagonistic phagocytic cells, resulting in persistent, latent and life threatening infections (Pinto-Alphandar *et al.*, 2000; Carryn *et al.*, 2003; Imbuluzqueta *et al.*, 2010; Xie *et al.*, 2014). The host organisms are pose with greater challenge as the body defense mechanism were not just infected but as well act as reservoir for the pathogenic organisms, while reaching and spreading the infection to the adjacent uninfected tissues (Pinto-Alphandary *et al.*, 2000). However, owing to their ability to escape the mammalian host phagocytic protection mechanism and establish a specialized intracellular milieu outside the host immune system, intracellular bacterial infection has remain a rising trend among humans and animals (Monack *et al.*, 2004; Steinberg and Grinstein, 2008). Several human infectious diseases such as leishmaniasis, brucellosis, tuberculosis, salmonellosis and histoplasmosis, are caused by intracellular microorganisms. They also caused opportunistic infections in immunosuppressed patients with AIDS or other conditions, where Mycobacterial infections are involved to cause more complications (Briones *et al.*, 2008; Monack *et al.*, 2004; Steinberg and Grinstein, 2008; Mehta, 1996; Peters *et al.*, 2000). Due to their opportunistic nature, no detailed explanations have been highlighted on the physiological adaptation mechanism to intracellular survival and replication strategies. Though, the continuous intracellular survival of these pathogens is not an important virulence factor in their life cycle (Van Bambeke *et al.*, 2006). Chronic infections are generally characterized by diverse changes in the intracellular microenvironment. Thus successful pathophysiological adaptation led to dormancy of specialized lymphoid tissues and prolonged or persistent invasion of the body by the pathogens (Ranjan *et al.*, 2012). In addition, chronic inflammation and autoimmune disorders are commonly associated with intracellular pathogens which usually participate in malignant processes (Kaufmann, 2011). Besides the famous facultative and obligate intracellular bacteria, several other common pathogenic bacteria are now recognized for intracellular survival under definite conditions (Andrade *et al.*, 2013).

Intracellular bacterial infections presents considerable challenges to antibiotic treatment, this was due to limited cellular penetration, and poor intracellular retention of the antibiotic in the phygocytes, thus imposing insignificant inhibitory or microcidal effects on the pathogens (Akbari *et al.*, 2013), (Drulis-Kawa and Dorotkiewicz-Jach, 2010). Consequently, the required intracellular drug levels for bacterial elimination are not met (Ranjan *et al.*, 2012). Such kind of infections have been connected with deterioration in health and treatment failure (Akbari *et al.*, 2013). Life-threatening infections are often caused by resistant intracellular bacteria, making them more difficult to treat. Treating patients with resistant intracellular strains, requires high doses of drugs which presents adverse effects to healthy organs and tissues (Andrade *et al.*, 2013).

Microbial resistance have become more complicated over time, greatly limiting antimicrobials success, and is an increasingly emergent crisis (Hajipour *et al.*, 2012). These include, among others impediments, reduces drug permeability, increased drug extrusion from cells, mutation at key antimicrobial-genes-binding sites and drug inactivation or modification by enzymes (Jayaraman, 2009; Deurenberg *et al.*, 2009; Périchon and Courvalin, 2009). Resistance to conventional antimicrobials is also ascribed to the alteration in microbial growth cycle, as well as decreased in bacterial susceptibility to antibiotics (Pinto-Alphandary *et al.*, 2000; Sandhiya *et al.*, 2009). Furthermore, bacteria express higher resistance to antibiotics by forming biofilms which provides protection for them. Thus, the reduced membrane permeability of bacteria is recognized as the main reason for antibiotic resistance (Jayaraman, 2009; Blecher *et al.*, 2011; Huh and Kwon, 2011; Davin-Regli *et al.*, 2008). With the emergence of multi-drug resistant bacteria, antibiotic resistance remain a top challenge for drug delivery against bacterial cells (Davin-Regli *et al.*, 2008).

Antibiotic delivery systems represent a promising solution for the challenges of intracellular bacterial infections and are alternative to conventional antibacterial therapy for efficient eradication of pathogens. In this regard, the use of antibiotics carrier systems may represent an incredible approach towards the treatment of intracellular bacteria. Antibiotic carrier systems can be endocytose and/or phagocytose in a similar manner with bacteria and then release into phagocytic cells carrying intracellular pathogens the drug payload (Briones *et al.*, 2008; Abed and Couvreur, 2014). In another development, foreign materials immediately after injection are subjected to opsonization. The same process by which bacteria surface molecules are tainted by opsonins and become more readily engulf by phagocytes. In this manner, the carriers system are recognized by the reticuloendothelial system (Pinto-Alphandary *et al.*, 2000).

The increased global assembly of engineered nanoparticles as impending drug carrier system necessitates a comprehensive understanding of their potential toxicity (Kroll-*et al.*, 2012). However, the clinical application of nanoparticles for diagnostic procedure and therapeutic purposes, imaging or as a delivery vehicles represent deliberate exposure to considerable dosage of the particles (Oostingh *et al.*, 2011). Many European Communities responsible for implementing laws have laid down a number of legislations concerning the use and exposure to nanoamterials (Commission of the European Communities, 2005; Commission, 2004). This has become indispensable as the general awareness of nanotechnology can be threaten by events such as "Germany 2006 nano scare", concerning the spray glass protective Magic-Nanoing (Ross, 2006), and the controversy of nanometer-sized sunscreen in the United States (Long *et al.*, 2006). Despite many reports on the toxicity of nanomaterials, the precise association between the engineered nanoparticles and the immune system have not been tentatively studied (Kroll *et al.*, 2012; Samberg *et al.*, 2010; McNeil *et al.*, 2007).

1.2 Problem Statement

Since the 1980s, flouroquinolones have been used in clinical practice, and they have contributed to major advances in the medical treatment of gram negative bacterial infections as frontline drugs (Pestova *et al.*, 2000; Pinto-Alphandary *et al.*, 2000). Active efflux from prokaryotes as well as eukaryotic cells strongly modulates the activity of this class of antibiotics (Van Bambeke *et al.*, 2000). Thus, the intracellular

flouroquinolones act hastily in a concentration-dependent manner but in an inadequate fashion and a sub-optimal/therapeutic way (Carryn *et al.*, 2003). This contributes to failure of conventional fluoroquinolones therapies as a result of decreased accumulation and poor retention of the antibiotics inside the cells (Jacoby, 2005; Briones *et al.*, 2008). Flouroquinolones resistance is often due to increased efflux (Singh *et al.*, 2011; Ahmed *et al.*, 2013). Over recent decades, the increasing emergence of antibiotic resistance in pathogenic bacteria has been worsening and resulted in severe and often lethal infections that cannot be treated by conventional therapy (Freire-Moran *et al.*, 2011).

Ciprofloxacin is one of the frequently used antimicrobial of the fluoroquinolones group available all over the world (Ambulkar *et al.*, 2009). It is required in several systemic diseases, having strong bactericidal effect against a broad range of clinically relevant gram-negative and gram-positive bacteria (Jain and Banerjee, 2008; Jeong *et al.*, 2008). Ciprofloxacin have been available only as conventional, immediate- release tablets, and has a biological half-life of about 3-5 hours for a single or repeated administration (Henry *et al.*, 2002; Bhalerao and Rote, 2012). Other factors limiting the success and clinical usage of ciprofloxacin includes poor solubility at physiological pH, which prevent it from diffusing into the lung fluid to instigate a therapeutic response at key affected site, bitter taste in solution, and rapid renal clearance, in which minimum of 70% of the oral dose is excreted unchanged in the urine (El-gendy *et al.*, 2010; Bhalerao and Rote, 2012). However, the frequent administration of ciprofloxacin is most associated with side effects such as central nervous system disorder and kidney problems (Spratto, 2012).

Whilst antimicrobial treatments usually involve a prolonged period of therapy, adequate antibiotic exposure is desirable to guarantee eradication of the microbial pathogens. Nevertheless, prolonged therapy is often associated with patient noncompliance, and incomplete treatment may result in the development of resistance. Poor compliance is exclusively a problem for drugs with short half-lives, since these drugs have short dosing intervals, and the number of doses require for microbial eradication is high (Gao *et al.*, 2011). Therefore, in order to attained a successful treatment, antibiotic must fulfill a series of criteria, including the ability to penetrate and be retained by the cell, the capacity to reach the intracellular target, and the display of activity against bacteria residing in the peculiar environment (Imbuluzqueta *et al.*, 2010). On the other hand, due to the deficient in new antibacterial agents, there is considerable interest in restoring the activity of older and conventional antimicrobials (Piddock *et al.*, 2010).

The use of safe and efficient delivery systems, capable of delivering therapeutic agents to sub-cellular levels is an ultimate goal in enhancing therapeutic effect. It is also a promising strategy in overcoming microbial resistance (Pelgrift and Friedman, 2013). It has recognized that extended-release drug formulation is beneficial in improving patient compliance, as regular administration can be avoided by maintaining stable and continuous plasma drug concentration over a prolonged period, and maximize the therapeutic effect of antibiotics while minimizing resistance (Blasi *et al.*, 2007; Gao *et al.*, 2011). Modifications in drug delivery to redirect the antibiotic from the circulation and target it to cells, tissues, or organs where infection occurs may lessen the chance for the flouroquinolones travels to bone and cartilage (Lee *et al.*, 2011). The encapsulation of antibiotics in carriers could avoid antibiotic efflux and enhance the drugs' intracellular retention, since delivery systems like nanoparticles are not

substrates of the efflux pump proteins (Plapied *et al.*, 2011). Moreover, encapsulation of antibiotic improve their pharmacokinetic by increasing serum half-life and decreasing apparent volume of distribution which can increases the maximum tolerated dose (Pinto-Alphandary *et al.*, 2000). Nanoparticles can be phagocytosed by host phagocytes containing intracellular microbes. Once inside host phagocytes, the antibiotic-nanoparticles delivery system could release high dose of the antibiotic to eliminate the intracellular microbes before developing resistance (Blecher *et al.*, 2011; Huh and Kwon, 2011; Huang *et al.*, 2011).

Many studies have reported the increased antimicrobial activity of ciprofloxacin-conjugated nanoparticles (Akbari *et al.*, 2013; Chono *et al.*, 2008; Hono *et al.*, 2007; Ong *et al.*, 2012). Likewise decreased antibiotic resistance was reported in the presence of Zinc Oxide nanoparticles (Banoee *et al.*, 2010). It is anticipated that the use of nanoparticles-based drug delivery systems will continue to improve treatment of bacterial infections and multidrug-resistant microbes (Huh and Kwon, 2011). However, no studies have been conducted on the potential of ciprofloxacin-conjugated biobased-cockle shells-derived calcium carbonate (aragonite) nanoparticles (CSCCAN), to enhance the efficacy of the drug. Moreover, calcium carbonate has been used for controlled delivery of biomolecules due to it biodegradability, biocompatibility, and porous nature with huge promises (Rodríguez-Ruiz *et al.*, 2013). CSCCAN has good physicochemical properties and a simple technique of preparation in a bulk-scale (Kamba *et al.*, 2013). The cockle shells (Anadara granosa), which is available in abundance, is often considered a waste (Mohamed *et al.*, 2012). A, porous aragonite calcium carbonate nanoparticles loaded with gentamicin sulphate with controlled released have been used successfully in ostoemyelitis treatment (Lucas-Girot *et al.*, 2005). Thus, calcium carbonate nanoparticles are expected to also enhance the efficacy of ciprofloxacin.

Human exposure to nanoparticles is inevitable as the particles become more widely used; hence nanotoxicology research is gaining attention. The challenge, however, is that the interaction of nanoparticles physicochemical properties with biological systems at the cellular level must be established prior to biomedical applications (Lewinski *et al.*, 2008; Shukla *et al.*, 2005; Kroll *et al.*, 2012; Oostingh *et al.*, 2011). Study conducted on the biocompatibility of cockle shells-derived calcium carbonate (aragonite) nanoparticles (CSCCAN) revealed their non-toxic effects and therefore considered potential agent for drug delivery (Kamba *et al.*, 2014). However, understanding the biological response of the nanoparticles at sub cellular and molecular level is crucial and can certainly present another line of information to appraise the interactions between the nanomaterials and cells.

1.3 Research Objectives

1.3.1 General objective

The main objective of the present study was to develop ciprofloxacin-cockle shells-derived Calcium carbonate (aragonite) nanoparticles (C-CSCCAN) as advanced vehicle for intracellular drug delivery and controlled released with good cytocompatibility, insignificant genotoxicity and high antibacterial efficacy.

1.3.2 Specific objectives

This research was carried out specifically:

i. To develop C-CSCCAN hybrid molecules and study its physicochemical properties, and delivery system
ii. To evaluate *in vitro* biological toxicity and immunogenic potential of C-CSCCAN and CSCCAN using macrophage (J7741.A) cell line.
iii. To evaluate the *in vitro* antibacterial activity of C-CSCCAN by identifying the diameter of inhibition zone and minimum inhibitory concentration and minimum bactericidal concentration against *Salmonella* Typhi.

1.4 Hypothesis

It is hypothesized that;

i. CSCCAN is an effective carrier for ciprofloxacin, improves the chemical and physical stability of the drug substance and could sustained release of the drug in the surrounding intracellular milieu.
ii. C-CSCCAN is biocompatible and nontoxic.
iii. The physicochemical properties of C-CSCCAN enhance susceptibility of intracellular *S.* Typhi, and augment the antibacterial performance of ciprofloxacin.

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