FABRICATION AND CHARACTERIZATION OF PLGA-MICROPARTICLE LOADED WITH ALPHA-MANGOSTIN

BY

AIMEN ABDO ELSAID ALI

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International Islamic University Malaysia

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ABSTRACT

Alpha-mangostin (AM) is a lipophilic material and has been reported to have multi-therapeutic activities. However, many obstacles have been documented that limit its medical applications. Therefore, this study aimed to encapsulate this compound in PLGA-based mico/nanoparticles using two different protocol namely single and double emulsion solvent evaporation techniques. Different experimental variables had been manipulated during the fabrication processes in order to investigate their effects on the particles' characteristics and to obtain optimized formulations for both employed protocol. Reverse-phase high performance liquid chromatographic method had also been developed and validated for their linearity, precision, accuracy, limit of detection and limit of quantitation for reliable quantification of AM content in the particles. In the double emulsion technique, levels of evaluated factors included polyvinyl alcohol (PVA) concentration, ratio of oil to aqueous phases and emulsification time for both primary and secondary emulsion were optimized by using response surface methodology (RSM). The optimized formula that was prepared by 1% PVA, 1:10 ratio of oil to aqueous phases, and sonicated at 2 and 5 min time for primary and secondary emulsions, respectively, showed an encapsulation efficiency of 39.1%, a particle size of 2.06 μm and with desirable polydispersity index value (0.95). Interestingly, this optimized formula had an aerodynamic diameter of about 784.3 nm that is desirable for pulmonary delivery. Thermal analysis of these microspheres showed a physical conversion of AM from crystalline to amorphous state due to strong hydrogen bonds formed between carboxylic groups of polymeric material and hydrogen groups of AM as shown by FTIR. Thereafter, influences of other factors including AM concentration and type of stabilizer were also studied on the particles features. The resultant microspheres were characterized for their encapsulation efficiency (EE), loading efficiency (LE), particle size (PS), polydispersity index (SPAN), external morphology, *in vitro* release profile and *in vitro* cytotoxicity against non-small lung cancer cells line (A549). Our data revealed that replacement of PVA with Tween 20 or Span 20 showed significant decrease in the EE at low AM concentration (1% w/v), while increasing the AM concentration up to 2.5% showed significant increment in LE. Addition of Tween 20 also improved both EE and LE percents. Most of AM-loaded microspheres exhibited cell inhibitory activities close to that of free AM despite their release profile showed sustained release of AM over four weeks with total cumulative release of about 36.9-44.3%. In single emulsion technique, the evaluated experimental variables were type of emulsification process, type of surfactant and PVA concentration. Our data revealed that Tween 20 and Span 20 were unable to stabilize water-in-oil (o/w) emulsion system at the ratio were 1:1 and ultrasonic processor had no disruptive effect on the AM stability. Optimized formula prepared with 1% PVA, 0.3% chitosan hydrochloride and emulsified by ultrasonic processor showed EE of 79.1%, LE of 26.4% and with a diameter of 156.6 nm (based on SEM. Cytotoxicity of these particles (IC_{50}; 24.6 μM) was lower than free AM (IC_{50}; 12.8 μM) that consistent with their cumulative release after same incubation time. Incorporation of chitosan modified zeta potential of the nanoparticles to positive charge. The fabricated particles may be used as promising nano/microcarriers system to deliver AM to the lung cancer tissue.

خلاصة البحث

الألفا مانجوستين (AM) هو مادة كارهه للماء وقد وثقت على احتوائها العديد من الفعاليات العلاجية. لكن هناك العديد من الدراسات وجدت أن هذه المادة تعاني الكثير من المشاكل التي بدورها تمنع استخدامها طبيا. لذلك تهدف هذه الدراسة الى حوصلة هذا المركب داخل جزيئات متناهية في الصغر مكونة من بوليمير ال(PLGA) باستخدام طريقتين مختلفتين هما طريقة المستحلب البسيط وطريقة المستحلب المعقد وكلتا الطريقتين تعتمدان على تبخير المذيب. العديد من العوامل التحضيرية قد تم تغيرها ودراسة تأثيرها على خواص الجزيئات الفيزيائية من أجل الحصول على تركيبة علاجية مناسبة. بالاضافة الى ذلك لقد تم تطوير وتوثيق طريقة تحليلية باستخدام جهاز ال(HPLC) لمعايرة كمية الألفامانجوستين المتحوصلة داخل هذه الجيزيئات. مستويات العوامل المدروسة في تقنية المستحلب المعقد والمكونة من تركيز ال(PVA) ونسبة معدل الوسط الزيتي للوسط المائي وزمن الاستحلاب لكلى المستحلبين الاولي والثانوي قد تم ضبطها باستخدام التصميم التجربي الذي يعتمد على علم التقنية السطحية الاستجابية (RSM). التركيبة المحسنة والتي حضّرت بتركيز (1%) من ال(PVA) وبمعدل عشر اضعاف من الوسط الزيتي الى واحد من الوسط المائي واستحلبت لمدة دقيقتين وخمس دقائق على التوالي لكل من المستحلب الاولي والمستحلب الثانوي قد أظهرت كفائة تغليفية قدرت ب(39.1%) وحجم جزيئي قدر ب(2.06µm) وبمعدل انتشار جزيئي مقبول. هذه التركيبة تمتلك أيضا قطر جزيئي هوائي قدر ب(784.3 nm) والذي يعتبر ضمن المجال المطلوب للتوصيل الرئوي. التحليل الحراري لهذه الجزيئات أظهر تحول فيزيائي لمادة ال(AM) من الحالة الكرستالية الى الحالة الغير متبلورة والتي نتجت من تكوين روابط هيدروجينية قوية بين المجموعات الكربوكسيلية للبوليمير والمجموعات الهيدروكسيلية لمادة ال(AM). بعد ذلك تأثير عوامل أخرى تشمل تركيز ال(AM) ونوع العامل المساعد على الاستحلاب قد تم دراستها على الخواص العامة للجزيئات. خصائص الجزيئات والتي تشتمل على الكفائة التغليفية (EE) والكفائة التحميلية (LE) وحجم الجزيئات (PS) ومعدل انتشار الجزيئات (SPAN) ومعدل التحرر للمادة المغلفة وسمّية هذه الجزيئات ضد الخلايا السرطانية الرئوية الكبيرة (A549) قد تم ايضا دراستها. النتائج أظهرت أن تبديل ال(PVA) ب(Tween 20) أو ب(Span 20) أدى الى تقليل ملحوظ في الكفائة التغليفية (EE) عند استخدام تركيز قليل من مادة ال(AM; 1%). بينما زيادة تركيز ال(AM) الى (2.5%) ادت الى زيادة ملحوظة في الكفائة التحميلية (LE). اضافة ال(Tween 20) كعامل مساعد على الاستحلاب حسّن من كلى الكفائتين التغليفية (EE) والتحميلية (LE). أغلب الجزيئات المحمّلة بمادة ال(AM) أظهرت فعالية سمّية ضد الخلايا السرطانية قريبة الى فعالية ال(AM) الغير مغلف بالرغم من أن معدل تحرر المادة الفعالة (AM) من هذه الجزيئات كان بطيئ واستمر الى أربع اسابيع بمعدل تحرر تراكمي كلي قدر بحوالي (36.9%-44.3%). في تقنية المستحلب البسيط العوامل التجريبية التي قيمت كانت نوع طريقة الاستحلاب ونوع العامل المساعد على الاستحلاب بالاضافة الى تركيز ال(PVA). النتائج الاولية أظهرت أن ال(Tween 20) وال(Span 20) غير قادرين على المحافظة على استقرار المستحلب البسيط (w/o). التركيبة المحسنة والتي حضّرت بتركيز (1%) من ال(PVA) وتركيز (0.3%) ال(chitosan) واستحلبت بواسطة الموجات الفوق صوتية أظهرت كفائة تغليفية (EE) قدرت ب(79.1%) وكفائة تحميلية (LE) قدرت ب(26.4%) وبقطر حجمي قيس ب(156.6 nm) اعتمادا على تقنية المايكروسكوب الالكتروني (SEM). سمّية هذه الجزيئات ضد الخلايا السرطانية كانت أقل بكثير من سمّية ال(AM) الغير مغلف وهذه السمّية تتوافق مع كمية المادة المحررة بعد نفس المدة الزمنية المستخدمة خلال قياس الفعالية السمّية. مزج ال(chitosan) مع هذه الجزيئات غيّر طاقتها الكهربائية الى شحنة موجبة. ان هذه الجزيئات قد تستخدم مستقبلا كنظام جزيئي حامل لمادة ال(AM) وتوصيلها الى خلايا السرطان الرئوية.

ABSTRAK

Alpha-mangostin (AM) adalah bahan lipophilic dan telah dilaporkan mempunyai pelbagai aktiviti terapeutik. Walau bagaimanapun, banyak halangan yang mengehadkan aplikasi perubatan bahan ini. Atas alasan itu, kajian ini dilakukan bertujuan untuk memuatkan kompaun ini dalam mikro/nanopartikel yang berasaskan PLGA menggunakan dua protokol yang berbeza iaitu method tunggal dan teknik emulsi penyejatan pelarut berganda. Kaedah HPLC juga telah dibangunkan dan kelinearan, ketepatan dan had pengesanan partikel yang dipercayai mengandungi AM. Dalam teknik emulsi berganda, faktor-faktor yang dinilai termasuklah kepekatan polivinil alkohol (PVA), nisbah minyak kepada fasa akueus dan masa pengemulsian emulsi telah dioptimumkan dengan menggunakan kaedah gerak balas permukaan (RSM). Formula yang optimum telah disediakan dengan menggunakan 1% PVA, nisbah 1:10 antara minyak dan fasa akueus, dan masa sonikasi pada 2 dan 5 min, masing-masing, menunjukkan kecekapan pengkapsulan pada tahap 39.1%, saiz partikel pada 2.06 μm dan dengan nilai indeks polidispersiti yang memuaskan (0.95). Menariknya, formula yang dioptimumkan ini mempunyai diameter aerodinamik kira-kira 784,3 nm dan sesuai untuk penghantaran melalui paru-paru. Analisis terma menunjukkan penukaran fizikal AM dari kristal kepada amorfus disebabkan oleh ikatan hidrogen yang kuat terbentuk di antara kumpulan karboksilik yang terdapat pada polimer dan kumpulan hidrogen AM seperti yang dibuktikan oleh FTIR. Selepas itu, pengaruh faktor-faktor lain termasuk kepekatan AM dan jenis penstabil juga pengaruh ke atas ciri-ciri partikel. Mikrosfera yang difabrikasi telah disifatkan berpandukan kepada kecekapan mereka mengkapsul, kecekapan muatan AM, saiz partikel, polidispersiti indeks, morfologi luaran, profil pembebasan untuk *in vitro* dan kadar toksik bagi *in vitro* terhadap sel-sel kanser paru-paru (A549). Data kami menunjukkan bahawa penggantian PVA dengan Tween 20 atau Span 20 menunjukkan penurunan yang ketara dalam kecekapan mengkapsul pada kepekatan AM yang rendah (1% w/v), manakala peningkatan kepekatan AM sehingga 2.5% menunjukkan peningkatan yang ketara dalam kecekapan muatan. Penambahan Tween 20 juga telah menambahbaikkan kedua-dua kecekapan mengkapsul dan kecekapan memuatkan partikel yang difabrikasi. Kebanyakan AM yang dimuatkan mempamerkan aktiviti perencatan sel dan profil pembebasan mereka menunjukkan pelepasan berterusan daripada partikel yang mengandungi AM melebihi empat minggu dengan jumlah pengeluaran terkumpul kira-kira 36,9% ke 44,3%. Dalam teknik emulsi tunggal, pembolehubah yang dinilai adalah sejenis proses pengemulsian, jenis dan kepekatan surfaktan PVA. Data kami menunjukkan bahawa Tween 20 dan Span 20 tidak dapat menstabilkan sistem emulsi air dalam minyak (o/w) dan pemprosesan ultrasonik tidak mempunyai kesan yang mengganggu kestabilan AM itu sendiri. Formula yang dioptimumkan disediakan dengan menggunakan 1% PVA, 0.3% kitosan hidroklorida dan pemprosesan ultrasonik menunjukkan kecekapan mengkapsul daripada 79.1%, kecekapan muatan daripada 26.4% dan dengan diameter partikel pada 156,6 nm (berdasarkan SEM). Kadar toksik partikel ini (IC50; 24.6 μM) adalah lebih rendah daripada sampel yang bebas AM (IC50; 12.8 μM). Penggunaan kitosan dalam formulasi ini telah mengubah potensi zeta partikel nano tersebut yang bercaj positif. Partikel yang dimajukan dilihat mampu digunakan sebagai pembawa yang bersaiz mikro yang boleh dihantar melalui sel kanser paru-paru.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion; it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in science (Pharmaceutical Technology).

.....
Farahidah Mohamed
Supervisor

.....
Muhammad Taher
Co-supervisor

I certify that I have read this study and that in my opinion; it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Science (Pharmaceutical Technology).

.....
Kausar Ahmad
Internal Examiner

.....
Mohd Cairul Iqbal Mohd Amin
External Examiner

This dissertation was submitted to the Department of Pharmaceutical Technology and is accepted as a fulfilment of the requirements for the degree of Master in Science (Pharmaceutical Technology).

.....
Juliana Md Jaffri
Head Department of
Pharmaceutical Technology

This dissertation was submitted to the Kulliyah of Pharmacy and is accepted as a fulfilment of the requirements for the degree of Master in Science (Pharmaceutical Technology).

.....
Siti Hadijah Binti Shamsudin
Dean, Kulliyyah of Pharmacy

DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Aimen Abdo Elsaid Ali

Signature …… Date ……..

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This thesis is dedicated to my beloved parents

for their endless assistance and encouragement

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LIST OF ABBREVIATIONS

AM	Alpha-mangostin
PLGA	Poly(D,L-Lactic-co-Glycolic acid)
EPR	Enhanced permeability and retention effect
PVA	Polyvinyl alcohol
RSM	Response surface methodology
CCD	Central composite design
FTIR	Fourier transform infrared spectrometer
EE	Encapsulation efficiency
LE	Loading efficiency
PS	Particle size
SPAN	Polydispersity index
NSCLC	Non-small cell lung cancer
w/o	Water-in-oil
w/o/w	Water-in-oil-in-water
IC_{50}	Minimum concentration can inhibit 50% of cell growth
SEM	Scanning electron microscope
DSC	Differential scanning calorimetry
LOD	Limit of Detection
LOQ	Limit of Quantitation
SD	Standard deviation
RSD	Relative standard deviation
DCM	Dichloromethane
EA	Ethyl acetate
T_g	Glass transition temperature
RP-HPLC	Reverse-phase high performance liquid chromatography
CH-HCl	Chitosan hydrochloride
CH-NPs	Nanoparticles modified with chitosan hydrochloride
GML	*Garcinia mangostana L*
PDA	Photodiode detector array
ROS	Reactive oxygen species
LDL	Human low density lipoprotein
TNF-α	Tumor necrosis factor-alpha
MIC	Minimum inhibitory concentration
DNA	Deoxyribonucleic acid
$\Delta\psi m$	Mitochondrial membrane potential
ATP	Adenosine-5-triphosphate
DPI	Dry powder inhaler
FDA	Food and Drug Administration
GIT	Gastrointestinal tract
ORAC	Oxygen radical absorbance capacity

CHAPTER ONE

INTRODUCTION

1.1. OVERVIEW

Cancer can be defined as a malignant disease characterized by excessive production of immature cells due to the impairment of vital mechanisms that command cell survival, proliferation and differentiation processes (Katzung, Masters, and Trevor, 2009). According to the American Cancer Society (2011), this malignant ailment had been statistically reported as the second leading cause of death in the United States of America. In particular, pulmonary neoplasm is considered as the main cause of cancer fatality in both men and women (Patricia, 2010), since it is responsible for approximately one million deaths annually over the world (Mok et al., 2011).

In Malaysia, lung cancer is collectively the third most common tumour among the citizens and the second most common tumour to afflict male (Sachithanandan & Badmanaban, 2012). Its mortality rate remained consistent with the global trend, since it represented about 19.8% of the overall death cases medically documented as caused by cancer (Sachithanandan & Badmanaban, 2012). It is worthy to note that the Malaysian government had spent more than RM 440 million annually in order to control the lung cancer spreading among the population and its associated problem, which burdened on the national health care finance (Sachithanandan & Badmanaban, 2012).

It is well-documented that cancer can arise due to a genetic disfigurement, which results from various internal and external factors that may act synergistically or in sequence to provoke carcinogenesis. Tobacco, chemicals, radiation and infectious

organisms such as hepatitis B virus (HBV), human papilloma virus (HPV), human immunodeficiency virus (HIV) and *Helicobacter pylori* are the most common external factors that caused cancer. Additionally, cancer may relate to internal factors such as hormones, inherited mutations and mutation that result from nutrition metabolism within the cells (Katzung et al., 2009). In particular, the lung cancer is mainly caused by cigarette smoking in addition to other vocations or ecological exposure to radon, asbestos, certain heavy metals; such as chromium, cadmium, arsenic and some organic chemicals, air contamination and perhaps a medical history of tuberculosis (Jemal, Siegel, Xu, & Ward, 2010). Recently, the World Health Organization (WHO, 2010) reported that smoking to be responsible for about 71% of lung cancer, 42% of chronic pulmonary diseases and approximately 10% of cardiovascular diseases (Su et al., 2012). In parallel with the global trend, the annual report of the Ministry of Health in Malaysia (MOH, 2002) had stated that smoking is the main cause of mortality in governmental hospitals, since it is responsible for nearly one third of the overall disease-related deaths (Su et al., 2012). Thus, cigarette smoking may play an important role as an etiological risk factor, and obviously more than 92% of the Malaysian male who suffer from lung cancer have a smoking history (Sachithanandan & Badmanaban, 2012). Therefore, control the cigarette smoking can be used as an effective preventable cause of lung cancer fatality (Patricia, 2010). However, it is estimated that if the cigarette abusers use to simultaneously stop smoking at the present time, it would require more than twenty years to observe an ultimate decline in mortality caused by this malignant disease (Sachithanandan & Badmanaban, 2012).

Overall, those carcinogenic factors cause deterioration of the genes, particularly “tumor suppressor genes”, which play a critical role in the discretion of neoplastic transformation (Katzung et al., 2009), and lead to production of cancerous

abnormal cells. These cells may possess the ability to attack other distant tissue in the body and colonize various organs through a process called metastasis. The metastatic process as well as series of metabolic malfunctions accompanied with the cancer may lead to tumor-related disorders and ultimate death of the patient unless the tumor can be effectively eradicated with suitable remedies (Katzung et al., 2009).

With respect to cancer therapy, a histological assessment of cancer cells and the tumor's clinical staging diagnosis using physical, laboratory and imaging examinations, are ultimately important to ensure effective eradication of cancerous tissue. From a histological point of view, bronchogenic carcinoma can be classified into two main groups; small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Based on the location of infected tissue, the later can be subdivided into squamous cell carcinoma (bronchial epithelium), adenocarcinoma (mucous gland), bronchioloalveolar cell carcinoma (epithelial cells of terminal bronchioles) and large cell carcinoma (miscellaneous group of undifferentiated tumor). These cells are generally characterized by their slowly spreading and therefore, they may only be treated in the early stages by surgical resection (Patricia, 2010). According to the recent screening study for lung cancer in Malaysia, about 88% of the total cases were histologically identified as non-small cell lung cancer (Sachithanandan & Badmanaban, 2012)

On the other hand, clinical staging of the tumor can be determined depending on the so-called TNM approach. This "international staging system" illustrates a physical appearance of the neoplasm, since the capital letters T, N and M refer to the size and place of the primary tumor, the occurrence and place of nodal metastases and the presence or disappearance of metastases, respectively. Consequently, the prognosis of tumor can be classified into four main categories namely; I (A&B), II

(A&B), III (A&B) and IV stages (Patricia, 2010). Unfortunately, most of lung cancer cases in Malaysia, which accounted for 75% of the total cases, were diagnosed at the distant stages; III and IV (Sachithanandan & Badmanaban, 2012). Lung cancer at the early stages is typically asymptomatic which reflects the delay in diagnosis (Siegel et al., 2012).

The surgical resection is undoubtedly involved in the treatment of NSCLC. It is noteworthy that numerous patients who were diagnosed at the early stages; I and II, can be effectively cured by surgical resection. However, under special conditions some patients with stage IIIA can benefit from surgical resection. In contrast, the resection would be unprofitable with patients who are diagnosed at the distant stages; IIIB and IV. Those patients are commonly treated with chemotherapy and radiotherapy in order to promote their survival (Patricia, 2010). It is interesting to note that chemotherapy is not a very efficient strategy to completely eradicate cancerous tissues as shown by the little increment in the overall survival in patients who had been diagnosed with stages IIIB and IV of NSCLC (Patricia, 2010). Generally, the current treatment strategy of malignant tumor is based on intrusive approach which commences with employing a catheter to allow chemotherapy and delivering an initial chemotherapeutic agent in order to diminish the tumor mass and then, surgical resection for the remaining cancerous tissues. Subsequently, the patient will be subjected to more chemotherapy and radiotherapy as prophylactic approaches to prevent the possible recurrence of cancer (Brannon-Peppas & Blanchette, 2012).

1.2. BACKGROUND OF THE STUDY

The treatment of cancer with a vast number of chemotherapeutic agents is limited by their serious side effects on the normal tissues. This limitation is reflected by the non-

specific distribution of most anti-neoplastic remedies through both normal and cancerous tissues (Brannon-Peppas & Blanchette, 2012), rapid excretion rate owing to their small molecular weight and thus, requiring continual and large dose administration (Amiji, 2006). For example, paclitaxel exhibits a potent anticancer activity against a broad spectrum of cancerous tissues especially NSCLC (Brannon-Peppas & Blanchette, 2012). However, due to its poor water-solubility, it was formulated using a co-solvent system composed of polyethoxylated castor oil (Chremophor EL) and ethanol (Long, 1994). The resultant product; Taxol® is the first developed product of paclitaxel that was employed as anticancer agent for treatment of NSCLC. Nevertheless, Cremophor EL is a toxic adjuvant and exerts many unwanted effects including hypersensitivity reaction, nephrotoxicity and neurotoxicity (Singla, Garg, & Aggarwal, 2002). Likewise, doxorubicin is another example of anti-neoplastic agent that has been widely employed for the treatment of cancer. It was found that doxorubicin in its free form exhibited various side effects such as cardiotoxicity and myelosupression (Brannon-Peppas & Blanchette, 2012). Consequently, an efficient therapeutic approach for cancer based on the development of a new agent that is able to specifically target the cancerous tissue is a challenge.

For the past decades, alpha-mangostin (AM) had been evaluated *in vitro* as a potent anticancer agent against diverse types of cancer cell lines especially NSCLC (Shih et al., 2010). In addition, it had been reported to exhibit a wide spectrum of biological activities such as anti-oxidant (Pedraza-Chaverri et al., 2009), anti-inflammatory (Chen, Yang, & Wang, 2008), analgesic (Cui et al., 2010), anti-fungal (Kaomongkolgit, Jamdee, & Chaisomboon, 2009) and anti-bacterial (Iinuma et al., 1996) effects. Although AM had been documented to exert a lower anti-proliferative activity against the NSCLC than Paclitaxel (13 μM versus 0.004 μM), it is interesting

to note that AM offer some unique features over the clinically-approved anticancer drugs, since AM has neuro-protective (Tangpong et al., 2011), cardio-protective (Devi & Vijayaraghavan, 2007) and nephro-protective (Perez-Rojas et al., 2009) properties. Nevertheless, the medical use of AM is limited because of its low oral bioavailability and rapid non-specific distribution into the tissues which are consequence of its hydrophobic nature.

Successful advanced drug delivery is basically a collective work of a multidisciplinary science including chemistry, engineering and biology. The technology allow us to modulate the delivery of drug molecules aimed to achieved optimal therapeutic concentration at a specific site of action for effective treatment (Uchegbu & Schätzlein, 2006). As such, here in this thesis, we had employed an aspect of advance drug delivery technology to design therapeutic particles that not only had the ability to provide controlled-release of therapeutic agent but also to specifically target the cancerous tissue with minimal side effect. The latter could be achieved by protecting the intact tissue from drug toxicity (Amiji, 2006) which we hope to obtain a therapeutic agent that can improve therapeutic outcome in the management of cancer treatment. Our scope of study is designing nanoparticles for passive targeting only and not active targeting, with pulmonary and intravenous as routes of drug administration.

In this study, to overcome the problem of exposing healthy cells to cytotoxic agent, alpha-mangostin was encapsulated into biodegradable PLGA-micro/nanoparticles. We aimed to design a particle that probably can later be formulated into suitable pulmonary and intravenous delivery. Passive targeting by pulmonary delivery can be achieved owing to aerodynamic diameter that determines deposition of the particle at different section of the lung. It is postulated that when