UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF A NANOPARTICLE-MEDIATED DELIVERY SYSTEM TO STUDY RELEASE OF FLUORESCENTLY-LABELED GLUTAMIC ACID ENCAPSULATED IN CHITOSAN NANOPARTICLES

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By

UMMU AFIQAH HASSAN

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

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DEDICATIONS

This thesis is especially dedicated to my beloved parents, Hassan Misman and Normala Ahmad for their endless love, understanding and prayers. To all my siblings, family members and friends, thank you for all your support and encouragement throughout entire of my postgraduate life.

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

DEVELOPMENT OF A NANOPARTICLE-MEDIATED DELIVERY SYSTEM TO STUDY RELEASE OF FLUORESCENTLY-LABELED GLUTAMIC ACID ENCAPSULATED IN CHITOSAN NANOPARTICLES

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Chair: Mas Jaffri Masarudin, PhD
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Inefficient cellular delivery and intracellular accumulation are major drawbacks towards achieving favorable therapeutic responses for many therapeutic drugs and biomolecules. To tackle this issue, nanoparticle-mediated delivery vectors have been aptly explored as a promising delivery strategies capable to enhance the bioavailability and cellular localization of biomolecules and therefore, improve their therapeutic efficacies. However, the dynamics of intracellular biomolecule release and accumulation from such nanoparticle systems has remain scarcely studied. Therefore, in this study, chitosan nanoparticle (CNP) was synthesized to serve as the delivery carrier for glutamic acid, a model for encapsulated biomolecules. Various chemical and morphological analyses were conducted to verify the nanoparticle formation and glutamic acid loading. In order to track the glutamic acid release and accumulation, the glutamic acid was then fluorescently-labeled with fluorescein isothiocyanate (FITC) prior encapsulation into CNP. This study therefore describes the encapsulation, release and accumulation of fluorescently labeled-glutamic acid from a robust, non-efficacious chitosan-based nanoparticle delivery system using tripolyphosphate (TPP) as a cross-linker. Light Scattering data concluded the formation of small-sized and monodispersed CNP at a specific volume ratio of chitosan to TPP. Following encapsulation, nanoparticle size increased exponentially to >100 nm to accommodate the glutamic acids within its core. Electron microscopy images revealed a discrete and spherical shape of CNP populations. The particle size increased over 100 nm following glutamic acids loading as reflected by Light Scattering data. Formation of CNP was further reflected by reduction in free amine groups in chitosan, and Fourier Transform Infrared detected peaks of functional groups belonging to both chitosan and TPP. Approximately 60% glutamic acid were efficiently encapsulated into CNP, which further suggested the potential of CNP as a drug delivery vehicle. Cell viability assay demonstrated a low toxicity property of CNP; conferring about 70% cell viability of 786-O cancer cells at the highest concentration used. *In vitro* tracking of glutamic acids release via fluorescence microscopy revealed a time-dependent release and controlled accumulation of fluorescently modified-glutamic acids from CNP into 786-O cells from 6 hours to 48 hours treatment points. The fluorescently-labeled glutamic acids was found to be release into cells as early as 6 hours post

treatment. The fluorescence was gradually increased at 24 hours later and persisted inside the treated cells up to 48 hours. Flow cytometry data demonstrated a gradual increase in intracellular fluorescence signal from 30 minutes to 48 hours post treatment with fluorescently-labeled glutamic acids encapsulated CNP. These results therefore suggested the potential of CNP system towards enhancing the intracellular delivery and release of the encapsulated glutamic acids as well as controlling their accumulation and retention over prolong period of time. This CNP system thus may serves as a potential candidate vector capable to improve the therapeutic efficacy for drugs and biomolecules in medical as well as pharmaceutical applications through the enhanced intracellular release and accumulation of the encapsulated cargo.

Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

PENGHASILAN SISTEM PENGHANTAR MELALUI NANOPARTIKEL UNTUK MENGKAJI PELEPASAN ASID GLUTAMIK BERLABEL YANG DI KAPSULKAN KE DALAM SISTEM NANOPARTIKEL KITOSAN

Oleh

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Ketidakcekapan penghantaran dan pengumpulan di dalam sel adalah penghalang utama untuk mencapai respons terapi yang diinginkan oleh kabanyakan ubatan dan biomolekul. Bagi mengatasi isu ini, penghantaran menggunakan nanopartikel sebagai vektor telah diteliti sebagai satu strategi penghantaran yang berpotensi mampu meningkatkan bioavailabiliti dan pengumpulan biomolekul di dalam sel dan seterusnya meningkatkan keberkesanan terapi. Walau bagaimanapun, kajian tentang pelepasan dinamik biomolekul dalam sel dari nanopartikel yang merupakan aspek yang penting untuk aktiviti terapeutik masih kurang dipelopori. Kajian ini seterusnya menjelaskan pengkapsulan, pelepasan dan pengumpulan asid glutamik berlabel daripada sistem nanopartikel kitosan yang kukuh dan efektif dengan tripolifosfat sebagai penyambung. Data penyerakan cahaya menunjukkan pembentukan saiz partikel yang kecil dan seragam pada nisbah tertentu isipadu kitosan kepada tripolifosfat. Asid glutamik dilabel dengan fluorescein isothiocyanate sebelum dikapsulkan ke dalam nanopartikel kitosan. Selepas pengkapsulan, peningkatan saiz nanopartikel menunjukkan pengkapsulan asid glutamik di dalam terasnya. Pemerhatian di bawah mikroskop elektron menujukkan nanopartikel kitosan berbentuk sfera. Saiz partikel tersebut meningkat melebihi 100 nm selepas pengkapsulan asid glutamik seperti yang ditunjukkan oleh data penyerakan cahaya. Pembentukan nanopartikel kitosan seterusnya ditunjukkan oleh pengurangan kumpulan amino di dalam kitosan dan Fourier Transform Infrared mengenalpasti kehadiran kumpulan fungsi kitosan dan TPP. Anggaran 60% asid glutamik yang digunakan telah dikapsulkan ke dalam nanopartikel kitosan yang seterusnya mencadangkan potensi nanopartikel kitosan sebagai penghantar ubatan. Ujian kebolehhidupan sel menunjukkan sifat toksik nanopartikel kitosan yang rendah dengan 70% sel 786-O hidup pada kepekatan kitosan dan tripolifosfat yang paling tinggi. Pemerhatian pelepasan *in vitro* asid glutamik melalui mikroskop pendarflour mencadangkan pelepasan yang bergantung kepada masa dan pengumpulan terkawal asid glutamik berlabel daripada nanopartikel kitosan ke dalam sel 786-O dari 30 minit sehingga 48 jam rawatan. Asid glutamik berlabel telah dilepaskan seawal 6 jam ke dalam sel. Kependarflouran meningkat selepas 24 jam dan berterusan sehingga 48 jam. Data

aliran sitometri menunjukkan peningkatan isyarat pendarflour secara berkadar dari 30 minit sehingga 48 jam dalam sel yang di rawat dengan asid glutamik berlabel yang dikapsulkan ke dalam nanopartikel kitosan. Hasil kajian ini seterusnya mencadangkan keupayaan sistem nanopartikel kitosan dalam meningkatkan penghantaran dan pelepasan asid glutamik ke dalam sel selain mengawal pengumpulan dan pengekalannya untuk jangka masa panjang. Sistem nanopartikel kitosan ini berpotensi sebagai penghantar yang mampu meningkatkan keberkesanan terapeutik ubatan dan biomolekul dalam bidang perubatan dan farmaseutikal melalui peningkatan pelepasan dan pengumpulan kargo yang dikapsul di dalam sel.

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APPROVAL

I certify that a Thesis Examination Committee has met on (date of viva voce) to conduct the final examination of Ummu Afiqah Hassan on her thesis entitled "Development of a Nanoparticle-Mediated Delivery System to Study Release of Fluorescently-Labeled Glutamic Acids Encapsulated in Chitosan Nanoparticles" 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 degree of Master of Science.

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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 Master of Science.The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
◦C	degree celcius
CO_2	carbon dioxide
CS	chitosan
CNP	chitosan nanoparticle
DLS	Dynamic Light Scattering
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EPR	enhanced permeability and retention
EDTA	ethylene diamine tetraacetic acid
FACS	Fluorescence Assisted Cell Sorting
FBS	fetal bovine serum
FESEM	Field Emission Scanning Electron Microscopy
fGA	fluorescently-labeled glutamic acid
fGA-CNP	fluorescently-labeled glutamic acid encapsulated chitosan nanoparticle
FITC	fluorescein isothiocyanate
FTIR	Fourier Transform Infrared
GA	glutamic acid
GA-CNP	glutamic acid encapsulated chitosan nanoparticle
h	hour(s)
HCl	hydrochloric acid
M	molar
µl	microliter
mg	milligram
ml	milliliter
MTT	methyl thiazol tetrazolium bromide
nm	nanometer
PBS	phosphate buffered saline
PDI	polydispersity index
pKa	Acid dissociation constant
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute (media)
SDS	sodium dodecyl sulphate
siRNA	small interfering ribonucleic acid
TEM	Transimission Electron Microscopy
TNBS	trinitrobenzene sulfonic
TPP	tripolyphosphate

CHAPTER 1

INTRODUCTION

1.1 Research background and problem statement

The efficacy of many drugs and therapeutic moieties is dependent towards their effective uptake and accumulation into its targeted area (Farokhzad and Langer, 2009). Thus, several factors such as *in vivo* persistence, biological barrier-crossing capacities, and precise local uptake in adequate concentrations are paramount for achieving meaningful therapeutic responses. Use of conventional pharmacotherapies have often been associated with poor *in vivo* stability, short *in vivo* persistence, decreased intestinal permeation, low cellular transport capacity and non-selective delivery which often compromise any biological efficacies (Allen and Cullis, 2004; Aungst *et al*., 1996). Although the discovery of candidate therapeutic compounds remain exponential, herein lies a need to develop a system to increase their cellular accumulation and uptake for maximum therapeutic efficacy.

Nanotechnology has been spurring great research tractions in both biological and medical fields; specifically for drug delivery applications (De Jong and Borm, 2008). Nanoparticle-based approaches for pharmaceutical and medical applications have been attributed as a novel platform to address and remedy limitations of conventional drug delivery strategy. The use of nanoparticles as efficient delivery carriers requires them to not only be able to permeate tissues and accumulate inside cell, but must be preferably capable to efficiently release the payloads into the cells. The capacity of nanotherapeutics to passively or actively accumulate into many different cell types has been well reported in few number of studies. At present, many classes of potent nanovectors such as liposomes, micelles, dendrimers and polymeric nanoparticles have been proposed for delivery of a multitude of therapeutic agents and biomolecules include gene, protein and peptide drugs. These nanosized carriers provide a mechanism for local delivery of therapeutic molecules while controlling the impact of side effects (Mahapatro and Singh, 2011). Nanoparticles have also been shown to possess unique properties and functions as delivery carriers owing to their 'size effects' which promote better penetration through various body compartments (Xu *et al*., 2007). However, although the successful internalization of these carriers have been demonstrated, a proposed mechanism towards intracellular cargo release upon cellular uptake remains unascertained. Additionally, the dynamic release properties of encapsulated biomolecules from such nanoparticles at the cellular level has also yet to be elucidated in literature. The therapeutic efficacy of drugs which dependent on their accumulation into cells requires that its efficient release and accumulation into cells to be assessed and confirmed. Therefore, there lies a need to study the release and localization of the encapsulated cargo for designing an efficient vector capable to improve drug potency through an efficient intracellular cargo release and accumulation.

In this study, chitosan-based nanoparticles were synthesized, characterized and used as a delivery carrier for an amino acid as a model biomolecule for encapsulation. In order to elucidate release mechanisms and dynamics of the system, the use of fluorescently-labeled glutamic acid as the cargo was synthesized. Chitosan nanoparticles were synthesized via ionic gelation routes and optimized for encapsulation of fluorescently-

labeled glutamic acid. The resulting biomolecule-encapsulated nanoparticles were then characterized using various physicochemical methods. The potential of this nanoparticle system towards enhancing intracellular amino acid accumulation and release were then subsequently assessed using an *in vitro* cell line model, 786-O kidney cancer cells. Fluorescence microscopy was utilized to monitor the time-based release profiles and localization of the fluorescently-labeled glutamic acid from the nanoparticle system. Fluorescence-Activated Cell Sorting (FACS) analysis was used to quantitatively assess the accumulation of fluorescently-labeled glutamic acid in 786-O cells, thus subsequently verify the observations from fluorescence microcopy.

1.2 Justification of study

Despite the extensive reports on the potential uses of nanoparticles as drug delivery carriers, the dynamic properties regarding the intracellular cargo release and accumulation from nanoparticles which are prerequisite for the efficient therapeutic actions has remains scarcely studied. This research is aimed to bridge this gap of knowledge, by studying the intracellular release of glutamic acid as an encapsulated cargo from chitosan-based nanoparticles. The findings of this study would provide a fundamental knowledge on cargo release profile from colloid nanoparticle system. The successful implementation of this nanoparticle-mediated delivery system will significantly lead to a potential enhanced delivery of peptide and protein drugs for medical as well as pharmaceutical applications.

1.3 Objectives

The research objectives outlined in this thesis include:

1. To develop a chitosan nanoparticle system for the efficient encapsulation of fluorescently-labeled glutamic acid;
2. To perform physicochemical and morphological characterizations of the resulting glutamic acid encapsulated chitosan nanoparticles; and
3. To visualize the release and accumulation of fluorescently-labeled glutamic acid from the nanoparticle system.

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