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ENHANCED FUNCTIONALITY OF MIRNA-ENCAPSULATED CHITOSAN NANOPARTICLES AS AN ANTI-MIGRATION AGENT FOR CANCER THERAPY

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By

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ENHANCED FUNCTIONALITY OF microRNA-ENCAPSULATED CHITOSAN NANOPARTICLES AS AN ANTI-MIGRATION AGENT FOR CANCER THERAPY

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Despite advanced treatments in cancer therapy, there are still high incidences and mortality rates for cancer globally with expectations of 23.6 million new cancer cases by 2030. An inherent drawback towards the success of current treatment is the increases metastasis of the primary tumours to other body parts in advanced stage cancers. Current therapeutics therefore could potentially target metastasis routes as a means for novel cancer treatment strategies. Recently, microRNAs (miRNA) have been utilized as a repressor molecule for metastasis, as it inhibits fundamental processes related to cellular and physiological pathway of the tumour at the mRNA level. However, therapeutic applications of miRNAs is impaired by premature degradation in the extracellular environment by endonucleases. Nanoparticles can therefore be used as delivery vehicles for miRNA to increase its cellular uptake, as they confer protection to the miRNA from extracellular enzymatic degradation. The aim of this study was to develop optimal parameters for the synthesis of chitosan nanoparticles (CNP) using chitosan (CS) and sodium tripolyphosphate (TPP), a drug carrier with high particle stability, low cellular toxicity, and robust preparation methods through ionic gelation method for effective encapsulation of plasmid precursor miRNA186 (ppmiR186). A CNP nanoparticle system was subsequently developed and characterized physico-chemically, followed by an evaluative assessment of the efficiency for synthesized ppmiRNA186-encapsulated chitosan nanoparticle (CNP-ppmiR186) system to decrease the metastasis of cancer cells *in vitro*. Physico-chemical analyses using TNBS assay showed a decrease in free amine group of CS with increasing TPP volume, an indicator for utilization of protonated amine groups in CS by anionic phosphate groups of TPP to form CNP. Subsequent Dynamic Light Scattering (DLS) analysis of the nanoparticles showed a 135% increased of particle size from 84.06 nm to 197.63 nm and PDI from 0.28 to 0.37 after incorporation of 100 µg/µl ppmiR186; an encapsulation efficiency of 48% to form CNP-ppmiR186. Through gel electrophoresis, encapsulation of ppmiR186 in CNP showed that CNP-ppmiR186 formed a neutrally charged moiety, as no band was separated compared to naked ppmiR186. Further morphological analysis using FESEM and TEM showed a spherical shape for both CNP and CNP-ppmiR186 and correlated accordingly to particle sizes measured through DLS. Additionally, important functional groups (amine, inorganic and organic phosphate) in each molecule of CS, TPP, ppmiR186, CNP and CNP-ppmiR186

were observed in the corresponding CNP-ppmiR186 nanoparticle system. The nanoparticle system was then treated to A549 lung cancer cells, and evaluated the efficacy of ppmiR186. The resulted CNP-ppmiR186 was delivered in A549 cells of non-small cell lung carcinoma (NSCLC) expressed the miRNA-186 gene cassette, as shown by the sequential expression of an upstream green fluorescent protein (GFP) gene in transfected cells. Cell scratch and cytotoxicity assays were then conducted to determine metastasis and proliferation abilities of cancer cells after treatment with the nanoparticles. Both CNP and CNP-ppmiR186 successfully hindered migration of A549 cells in cell scratch assays, as the scratch gap on the monolayer cell only decreased by 1% and 4%, respectively compared to non-treatment groups. Finally, anti-proliferative effect of the CNP-ppmiR186 determined through MTT assay showed a 59% cell viability in cells treated. Moreover, CNP was ascertained as a safe delivery vehicle, as 68% cell viability was achieved even at its highest concentration of treatment. In conclusion, based on physiochemical analysis and cellular treatments, ppmiR186 was successfully encapsulated in CNP and the resulting CNP-ppmiR186 is suggested to possess enhanced anti-metastatic and anti-proliferative effect on A549 cells of NSCLC compared to naked delivery of ppmiR186. This system thus has the potential to be further developed as a novel cancer therapy preventing metastasis in cancers, and towards future aversion of cancer progression in patients.

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

PENGKAPSULAN microRNA DALAM PARTIKEL NANO KITOSAN YANG DIPERTINGKAT KEFUNGSIANNYA SEBAGAI AGEN ANTI-MIGRASI UNTUK TERAPI KANSER

Oleh

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Walaupun dengan pelbagai rawatan kanser yang maju, kadar kejadian dan kematian yang disebabkan oleh kanser masih tinggi dengan jangkaan 23.6 juta kes kanser baru pada tahun 2030. Penyembuhan dari rawatan kanser yang berjaya digagalkan oleh penyebaran atau metastasis kanser ke bahagian badan lain yang juga merupakan kanser tahap bahaya. Oleh itu, rawatan terbaru yang dapat terus menghentikan metastasis diperlukan sebagai salah satu rawatan kanser. Baru-baru ini, mikroRNA (miRNA) telah digunakan sebagai molekul penahan kepada metastasis tumor apabila ia menghalang proses asas berkaitan sel dan fisiologi tumor pada peringkat mRNA. Walaubagaimanapun, aplikasi teraputik oleh miRNA digagalkan oleh degradasi pramatang endonuklease di dalan persekitaran luar sel. Oleh itu, nanopartikel boleh digunakan sebagai pembawa miRNA untuk meningkatkan kadar kemasukan oleh sel kerana ia memberi perlindungan kepada miRNA daripada degradasi enzim di luar sel. Kerana itu, tujuan kajian ini adalah untuk membangunkan parameter optimum dalam menghasilkan nanopartikel kitosan (CNP) menggunakan kitosan (CS) dan natrium tripoliphosphate (TPP) sebagai pembawa ubat dengan kestabilan partikel yang tinggi, kurang kadar toksik pada sel dan cara penyediaan yang teguh melalui kaedah gel ionik untuk pengkapsulan plasmid *precursor* miRNA186 (ppmiR186) yang berkesan. Pengkapsulan ppmiR186 di dalam kitosan nanopartikel (CNP-ppmiR186) yang berjaya dihasilkan kemudiannya dicirikan secara fisiokimia dan penilaian kecekapan untuk mengurangkan metastasis sel kanser secara *in vitro.* Analisis fisiokimia melalui asai TNBS menunjukkan keadar pengurangan proton kumpulan amina CS dengan penambahan isi padu TPP dalam penghasilan sistem nanopartikel. Teknik penyerakan bercahaya dinamik (DLS) untuk mengukur saiz dan nilai PDI *(monodispersity)* nanopartikel menunjukkan kenaikan saiz partikel sebnayak 135% daripada 84.06 nm kepada 197.63 nm dan PDI 0.28 kepada 0.37 selepas inkorporasi 100 µg/µl ppmiR186 dengan 48% keberkesanan pengkapsulan CNP-ppmiR186. Melalui elektroforesis gel, pengkapsulan ppmiR186 di dalam CNP dibuktikan dengan ketiadaan jalur yang diasingkan berbanding ppmiR186 yang terdedah. Seterusnya, analisis morfologi melalui FESEM dan TEM menunjukkan bentuk sfera CNP dan CNP-ppmiR186 yang dihasilkan berbeza dengan bentuk molekul lain dan saiz yang diukur mempunyai persamaan saiz yang diukur terlebih dahulu menggunakan DLS. Sebagai tambahan, *functional groups* (amina, fosfat organik dan tak organik) dalam setiap

molekul CS, TPP, dan ppmiR186 disahkan diukur dalam sistem nanopartikel yang berkaitan. Sistem nanopartikel kemudiannya dirawat pada sel kanser paru-paru A549 untuk dinilai sebarang keberkesanan. Penghantaran CNP-ppmiR186 ke dalam sel besar karsinoma paru-paru disahkan melalui gen cahaya hijau pendarfluor yang diekspresikan. Kemudiannya, asai calar sel dan asai kesitotoksikan digunakan untuk kepastian keupayaan metastasis dan proliferatif kanser sel selepas dirawat dengan system nanopartikel. Melalui asai calar sel, kedua-dua CNP dan CNP-ppmiR186 menghalang migrasi sel A549 selepas jurang calar dihasilkan pada lapisan mono sel dengan kadar pengurangan jurang calar pada 1% dan 4% apabila dibandingkan dengan kumpulan yang ditidak dirawat. Akhir sekali, kesan anti-proliferatif CNP-ppmiR186 ditentukan melalui asai MTT dengan hanya 59% sel hidup dikesan. Tambahan pula, CNP juga disahkan sebagai pembawa ppmiR186 yang selamat kerana membolehkan sehingga 68% sel hidup walaupun pada kepekatan CNP tertinggi. Sebagai konklusi, berdasarkan kesemua analisis fisiokimia dan rawatan sel, ppmiR186 berjaya dikapsulkan di dalam CNP dan CNP-ppmiR186 yang dihasilkan mempunyai kesan anti-metastasis dan anti-proliferatif pada sel A549 dan seterusnya mempunyai potensi untuk dibangunkan sebagai terapi untuk menghalang metastasis kanser dan ke arah pembunuh kanser dalam pembangunan pesakit kanser yang lebih berjaya.

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I certify that a Thesis Examination Committee has met on (date of viva voce) to conduct the final examination of Syazaira Arham Binti Yahya Ariff on her thesis entitled Investigating the Enhanced Functionality of microRNA-Encapsulated Chitosan Nanoparticles as an Anti-Metastatic Agent for Cancer Therapy 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 in Nanobiotechnology.

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A549	Adenocarcinomic human alveolar basal epithelial cell line
A_{570}	Absorbance at 570 nm
A_{370}	Absorbance at 370 nm
A_{335}	Absorbance at 335 nm
BamHI	Bacillus amyloliquefaciens
bp	Base pair
CDK2	Cyclin-dependant kinase 2
CDK6	Cyclin-dependant kinase 6
Cells/ml	Cells per mililitre
CNP	Chitosan nanoparticle
CNP-ppmiR186	Chitosan nanoparticle encapsulated precursor plasmid microRNA 186
CS	Chitosan
DD	Degree of deacetylation
DNA	Deoxyribonucleic acid
DLS	Dynamic light scattering
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FTIR	Fourier transform infrared spectroscopy
FESEM	Field emission scanning electron microscopy
GFP	Green fluorescent protein
HCL	Hydrochloric acid
LB	Luria-Bertani
miRNA/miR	microRNA
miR186	microRNA 186
MMP2	Matrix Metalloproetinase 2
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaOH	Sodium hydroxide
NH_2	Amine
N-ppmiR186	Naked delivery of precursor plasmid microRNA 186
NSCLC	Non-small cell lung carcinoma
PBS	Phosphate buffered saline
ppmiR186	Precursor plasmid microRNA 186
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROCK1	Rho-associated protein kinase 1
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SEM	Standard error mean
shRNA	Short hair pin RNA
siRNA	Small interfering RNA
TAE	Tris Acetate-EDTA
TEM	Transmission electron microscopy

TNBS	Trinitrobenzene sulfonic assay
TPP	Sodium tripolyphosphate
T-ppmiR186	*Trans*IT-X2 Dynamic Delivery System for precursor plasmid microRNA 186
v/v	Volume per volume
w/v	Weight per volume
1Kb	1 kilo base

CHAPTER 1

INTRODUCTION

1.1 Research Background

There were 14.1 million new cancer cases reported in 2012 with lung cancer as the leading cause of incidence and mortality worldwide. By 2030, the number is expected to rise to 23.6 million (International Agency for Research on Cancer and Cancer Research UK, 2014). In Malaysia alone, there is an increase of 37.74% of incidence rates for cancer between 2007 and 2012, with lung cancer among the ten leading cancers after breast and colorectal cancer (Ariffin & Nor Saleha, 2011). The spread of cancer cells from one site to another, or metastasis causes cancer to be most deadly. Metastasized cancers commonly indicates a latent stage in which it is harder to be cured, with less survival rates compared to patients with local tumours. The National Cancer Institute has recorded 57% of cases of metastasized cancer with only 4.3% of survival rate for metastasized lung and bronchus cancer (Howlader *et al.,* 2016). It is therefore very desirable to develop cancer therapeutics that specifically stop cancer metastasis thus increase chances for successful cancer treatments and survival rate.

Recently, the development of ribonucleic acid interference (RNAi) in silencing cancer-causing genes is gaining much attention as a new approach in cancer therapy. MicroRNA (miRNA) as one of RNAi has gained much attention by researchers as a tumour suppressor and oncogene. Specifically, miRNA-186 (miR186) which is present in *Homo sapiens* play a critical role in non-small cell lung carcinoma (NSCLC); where its expression is downregulated involving multiple targeted genes, while upregulation of the miRNA has been suggested to suppress NSCLC growth. For example, cell cycle regulators cyclin D1, cyclin-dependant kinase (CDK) 2 and CDK6 are simultaneously targeted and suppressed by miR186 has been found in causing cell cycle arrest of NSCLC (Cai *et al.,* 2012). In another finding, overexpression of miR186 targeting Rho-associated protein kinase 1 (ROCK1) caused inhibition of NSCLC tumour growth and metastasis (Cui *et al.,* 2014). Through these literatures, the downregulation of endogenous miR186 expression leads to tumour cell invasion and delivering exogenous miR186 into target cells is an approach to counter abnormal growth and metastasize of NSCLC. However, its naked delivery is marred by low stability *in vivo* (Rothschild, 2014), thus a good delivery vehicle is requisite for effective administration of miR186 as a therapeutic option for cancer.

In this study, nanoparticle was used as the delivery vehicle to increase cellular uptake of the miR186 as it was protected from extracellular enzymatic degradation. Chitosan nanoparticles (CNP) has gained much attention as a valuable tool for novel drug delivery system utilized the interaction of chitosan (CS) and sodium tripolyphosphate (TPP) through ionic gelation method form the nanoparticles that will be used as the delivery vehicles. TPP was chosen as the cross linker with chitosan as it carries the most number of negative charges (five) compared to phosphate (four) and pyrophosphate (three). Higher number of negative charges resulted in higher ability of the anions to ionically

cross-link with cation of chitosan (Shu and Zhu, 2002). TPP has also been used extensively as the common cross-linker to form composed of chitosan, such as CNP. CNP is an example of a polymer-based organic nanomaterial which offers simpler preparation routes with safer assurance in biological environments with enhanced delivery and effectiveness compared to inorganic nanomaterials that need to undergo several surface modification prior to *in vivo* and *in vitro* induction.

This work demonstrated the use of CNP in delivering a precursor plasmid encoding the microRNA miRNA-186 (ppmiR186) as an anti-proliferation and anti-migration on NSCLC. Thus, the general objective of this study was to investigate the use of CNP as the delivery vehicle for the ppmiR186, tested against NSCLC cells, and evaluated to test its efficacy. Thus, the nano-mediated delivery system for the ppmiR186 was hypothesized to increase the ppmiR186 cellular uptake in NSCLC and decrease its cell invasion capabilities.

1.2 Objectives

The objectives set forth for this study were:

1) To develop optimal parameters of chitosan nanoparticle (CNP) synthesis for effective encapsulation of precursor plasmid miR186 (ppmiR186).
2) To perform physiochemical characterization of the CNP, and ppmiR186-encapsulated CNP, CNP-ppmiR186.
3) To evaluate the ppmiR186-encapsulated CNP and CNP system compared with ppmiR186 alone in suppressing cancer cell migration *in vitro*.

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