UNIVERSITI PUTRA MALAYSIA

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By

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

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GENE DELIVERY POTENTIAL OF BIO FUNCTIONAL CARBONATE APATITE NANOPARTICLE FOR LUNG CELLS

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Chairman : Associate Professor Syahril Abdullah, D.Phil

Faculty : Medicine and Health Sciences

Gene therapy has propelled innovations in therapeutic intervention of pulmonary related genetic diseases and cancer. However, lack of effective gene carrier system remains a major challenge in its clinical application. Carbonate apatite (CO_3Ap) nanoparticles have been assessed in various gene delivery trials in mammalian cells with promising results. However, no study has been performed on the gene delivery potential of CO_3Ap to lungs to date. Hence, this study aimed (1) to assess the gene delivery efficiency of CO_3Ap *in vitro* and in mouse lungs via intranasal delivery, (2) to evaluate the cytotoxicity effect of CO_3Ap/pDNA on the transfected lung cells and (3) to characterise the CO_3Ap/pDNA complex formulations.

Significantly high level of reporter gene expression was detected from lung cell line transfected with CO_3Ap/pDNA complex prepared in medium with and without serum. Further transfection study using CO_3Ap/pDNA complex formulation in 100 μl serum free medium was investigated. This pre-dosing study aimed to mimic the amount of the complex formulation a mouse lung

can accommodate. The reporter gene expression was found to be significantly higher than all the control groups when 8 µl of $CaCl_2$ was used to prepare the CO_3Ap [CO_3Ap(8µl)/pDNA]. Cytotoxicity analysis revealed that CO_3Ap/pDNA was not toxic to the cells. The percentage of the viable cells was found to be almost similar to the untreated cells. As for the characterization analyses, it was found that (1) the CO_3Ap/pDNA complex possesses aggregated spherical structure, particularly at higher concentration of $CaCl_2$ in the CO_3Ap formulations, (2) the CO_3Ap/pDNA complex was within nanometer range, (3) the sizes of the CO_3Ap particles were found to be proportional to the amount of $CaCl_2$ in the CO_3Ap formulations, (4) the CO_3Ap/pDNA possesses negative surface charge and the charge densities tended to be more negative when high amount of $CaCl_2$ in the CO_3Ap was used in the formulations, and (5) the CO_3Ap generated with 8 µl of 1M $CaCl_2$ [CO_3Ap(8µl)] offered considerable protection of pDNA against degradation by a nuclease.

In the lung of BALB/c mice, highest level of transgene expression was observed when CO_3Ap(8µl) was complexed with 40 µg of pDNA at day 1 post administration. Although massive reduction of gene expression was seen after day 1 post-administration, the levels remain significant through out the study time points. This indicates a prolonged gene expression property of the CO_3Ap/pDNA in the mouse airways.

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Oleh

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Terapi gen telah mendorong inovasi dalam rawatan terapeutik penyakit genetik berkaitan pulmonari dan juga barah. Walau bagaimanapun, kekurangan sistem pembawa gen yang berkesan masih menjadi cabaran besar dalam pengaplikasian terapi gen secara klinikal. Partikel nano karbonat apatit (CO_3Ap) telah dinilai dalam pelbagai ujian penyampaian gen kepada sel mamalia dengan keputusan yang memberangsangkan. Akan tetapi, setakat ini tiada kajian dijalankan terhadap potensi penyampaian gen oleh CO_3Ap kepada paru-paru. Oleh itu, kajian ini bertujuan untuk; (1) menilai keberkesanan penyampaian gen CO_3Ap secara *in vitro* dan kepada paru-paru mencit melalui rongga hidung, (2) menilai kesan toksik CO_3Ap/pDNA terhadap sel paru-paru yang ditransfeksi, dan (3) mencirikan formulasi kompleks CO_3Ap/pDNA.

Kadar ekspresi gen pelapor yang tinggi telah dicapai daripada titisan sel paru-paru yang telah ditransfeksi dengan kompleks CO_3Ap/pDNA yang disediakan dalam medium dengan serum dan bebas serum. Kajian transfeksi lanjut dijalankan menggunakan formulasi kompleks CO_3Ap/pDNA di dalam 100 μl medium bebas serum. Ini bertujuan mereplikasi jumlah formulasi kompleks yang boleh ditampung oleh paru-paru mencit. Ekspresi gen pelapor didapati lebih tinggi secara signifikan berbanding dengan kumpulan kawalan apabila 8 μl $CaCl_2$ digunakan untuk penghasilan CO_3Ap [CO_3Ap(8μl)/pDNA]. Analisa kesan toksik menunjukkan bahawa CO_3Ap/pDNA tidak toksik terhadap sel tersebut. Peratusan sel hidup didapati hampir sama dengan peratusan sel tidak diuji. Bagi analisa pencirian; (1) kompleks partikel CO_3Ap/pDNA didapati berstruktur sfera bergumpal, terutamanya pada kepekatan $CaCl_2$ yang lebih tinggi dalam formulasi CO_3Ap, (2) keseluruhan formulasi kompleks CO_3Ap/pDNA bagi kajian *in vitro* didapati bersaiz dalam julat nanometer, (3) saiz partikel CO_3Ap didapati berkadar langsung dengan jumlah $CaCl_2$ dalam formulasi CO_3Ap, (4) CO_3Ap/pDNA mempunyai cas permukaan negatif dan ketumpatan cas lebih cenderung negatif dengan penambahan $CaCl_2$ dalam formulasi CO_3Ap, dan (5) CO_3Ap yang dihasilkan dengan 8 μl 1 M $CaCl_2$ [CO_3Ap(8μl)] menawarkan perlindungan bagi pDNA terhadap degradasi nuklease.

Di dalam paru-paru mencit BALB/c, aras ekspresi transgen paling tinggi diperhatikan apabila CO_3Ap(8μl) dikomplekskan dengan 40 μg pDNA pada hari pertama selepas penyampaian gen. Walaupun ekspresi gen

diperhatikan berkurangan dengan mendadak selepas hari pertama gen disampaikan, aras ekspresi kekal signifikan sepanjang masa kajian. Ini menunjukkan sifat pemanjangan tempoh ekspresi gen oleh CO_3Ap/pDNA di dalam paru-paru mencit.

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I certify that a Thesis Examination Committee has met on 30TH of May 2013 to conduct the final examination of **Suleiman Yusuf Alhaji** on his thesis entitled "Gene Delivery Potential of Bio Functional Carbonate Apatite Nanoparticles for Lung Cells" 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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DECLARATION

I declare that the thesis is my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or currently submitted for any other degree at Universiti Putra Malaysia or any other institution.

_____

Suleiman Yusuf Alhaji

Date: 30th May, 2013

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LIST OF ABBREVIATIONS

A1AT	alpha 1-antitrypsin
ACUC	animal Care and Use Committee
ANOVA	analysis of variance
bp	Base pair
BSA	bovine serum albumin
CMV	cytomegalovirus
CO_2	carbon dioxide
CO_3Ap	carbonate Apatite
COPD	chronic obstructive pulmonary disease
CF	cystic fibrosis
D-SPM	dextran-spermine
DMEM	dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FESEM	field emission scanning electron microscope
GL67	Genzyme Lipid
h	hour
HCl	hydrochloric acid
IVC	individually ventilated cages

kDa	kilodalton
LB	Luria-bertini
M	molar (mol/liter)
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mM	millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
mV	millivolt
Na_2CO_3	sodium carbonate
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometer
PBS	phosphate-buffered saline
pDNA	plasmid DNA
PEI	polyethylenimine
PEG	polyethylenine glycol
PLGA	*poly(lactic-co-glycolic acid*)
RLB	Reporter Lysis Buffer
RLU	relative Light Unit
rpm	revolutions per minute
SD	standard deviation
SEM	standard Error of Mean
SEM	scanning electron microscope

TAE	Tris acetate EDTA
UV	ultraviolet
V	volt
µg	microgram
µl	microliter
µm	micrometer

CHAPTER 1

INTRODUCTION

Gene therapy holds the potential of therapeutic intervention for pulmonary related diseases such as asthma, emphysema, cystic fibrosis and lung related cancer. In general, gene therapy involves replacing, manipulating or augmenting defective or diseased genes with therapeutic gene with the aim of achieving cure. It requires a suitable and efficient carrier, either as non-viral or viral, to deliver the healthy gene to the lung cells for genetic correction. The therapeutic gene is believed to improve the physiological functions of the defective cells in the lung, once the gene expresses the expected protein. Pulmonary epithelium is comparatively accessible via the airways and that makes it an attractive candidate for gene therapy.

Although numerous non viral carrier systems have been examined in lung gene delivery, including cationic polymer of dextran spermine (Abdullah *et al*., 2010), polyethylenimine conjugates (Wong *et al*., 2006) and cationic lipid, such as lipoplexes and polyplexes (Ding *et al*., 2008), the majority failed to provide outstanding results for standard requirements in clinical application. This is mostly influenced by their inefficiency in gene delivery, cytotoxicity effect and ineffectiveness in targeting the desired organs or tissues in the body.

The current understanding of genetic approaches in the treatment of lung diseases reveals that enhancing transportation of gene into the airway cells

is a critical step for improving gene therapy. The physical barriers to the airway gene transfer and the enzymatic activity in the lung that may render the gene transfer ineffective have to be overcome. Lately, non-viral gene delivery system in form of nano-particles has emerged as an exciting alternative to the conventional non-viral systems for gene therapy of many genetic diseases. It has been reported that therapeutics nucleic acid complexed with bio functional carbonate apatite (CO_3Ap) nano-particles have remarkable properties capable of mediating high levels of gene delivery *in vitro.* This is due to the biodegradability, strong affinity for DNA and biocompatibility of the CO_3Ap. Previous studies showed that these particles possess high dissolution rate in endosomal acidic pH, leading to the rapid release of the bound DNA for high levels of protein expression in various cell lines (Chowdhury, 2007). Moreover, because of their nano-size dimensions and sensitivity to low pH, they can be quickly degraded when taken up by the cells in their acidic vesicles, without any indication of toxicity. These unique properties hold the promise for its applications in the mammalian cells.

Studies on carbonate apatite mediated gene delivery to HeLa cells demonstrated significant luciferase reporter gene expression compared to lipofection and calcium phosphate co-precipitation (Chowdhury & Akaike, 2007). Outstanding expression efficiency was observed in NIH 3T3 cells, which resulted in over 50-times higher expression when compared to the existing conventional methods. Transgene expression was also significantly higher in mouse primary hepatocytes (Chowdhury *et al.,* 2006).Efficient reporter gene expression from pEGFP-N2 in embryonic teratocarcinoma

stem cells utilising CO_3Ap nanoparticles as the gene carrier system has been reported (Kutsuzawa, Akaike, & Chowdhury, 2008). The study also suggested that the addition of specific membrane protein to the CO_3Ap could enhance the delivery, and consequently improved the expression of the gene in the embryonic teratocarcinoma cells. This development provides an exciting hope for the use of CO_3Ap gene delivery system in stem-cell-based therapy.

Polyethylenimine (PEI) perhaps is the most active and most studied cationic polymer for gene delivery to the lung. Chemically, PEI is one of the most densely charged polymers, having one third of its atoms composed of nitrogen, and one sixth of the nitrogen atoms carry a positive charge at physiological pH (Tang *et al*., 1997). Both linear PEI (LPEI) and branched PEI (BPEI) have excellent transfection activities *in vitro*, but exhibit moderate gene delivery activity *in vivo* (Di Gioia & Conese, 2008). However, the non-biodegradability nature of PEI makes it not suitable for safe transfection, as it is known that the toxicity and transfection activity of PEI is molecular weight–dependent. Chronic lung related diseases such as cystic fibrosis and lung cancer require long-term therapeutic effects. Unfortunately; the therapeutic window for gene therapy using non-viral gene transfer system is transient. This short-lived therapeutic effect is due to the non-integrative nature of the exogenous gene to the host genome. Repeated administrations of the gene therapy formulations can be employed to achieve sustained treatment. Yet, lung epithelial cells tend to be slowly dividing or terminally differentiated (Rawlins & Hogan, 2008). Therefore, prolonged and repeated use of PEI gene transfer system in order to achieve a sustainable therapeutic

expression of the gene is highly impractical due to its questionable safety profiles.

Although CO_3Ap nanoparticles as non-viral vector have been proven to be effective in the delivery of genes and therapeutics into various cell types *in vitro*, no study has been performed to elucidate the efficiency of this nano-sized crystal in the lung to date. This leads to the **general objective** of this study, which is to assess the transfection efficiency of the bio functional CO_3Ap nanoparticles plasmid DNA (pDNA) complex in the lung cells and mouse lungs. It is **hypothesized** that CO_3Ap is an efficient gene delivery system to the lung cell lines and can be subsequently used for gene transfer to the mouse lung.

In this study, the ability of the CO_3Ap to deliver gene *in vitro* was assessed first. This enabled us to properly understand the gene delivery, expression efficiency and toxicity of the complex formulations on the lung cells. The delivered gene to the cells is in the form of Luciferase reporter gene, which is designed to express detectable protein that can be easily analysed in the laboratory. The following analysis focused on the physical characteristics of the CO_3Ap at its optimal gene expression conditions based on its size and the pDNA protection levels offered by the formulations against nuclease activity. The final study assessed the most optimal conditions for gene expression in the mouse lungs.

The specific objectives of the study are as follow:

1) To optimise the CO_3Ap/pDNA formulations that can best transfect lung cells *in vitro*.
2) To investigate the cytotoxicity of the CO_3Ap/pDNA complex formulation *in vitro*.
3) To analyse the characteristics of the CO_3Ap/pDNA that give the best transfection efficiency *in vitro*.
4) To determine the gene delivery potential of the CO_3Ap in the lungs of mouse based on its formulations, amount of pDNA complexed, and time-pointpost administration.

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