UNIVERSITI PUTRA MALAYSIA

TARGETED DELIVERY OF SHORT HAIRPIN RNA EXPRESSING PLASMID USING HEPATITIS B VIRUS-LIKE PARTICLE FOR Bcl-2 GENE SILENCING IN CERVICAL CANCER CELLS

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By

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TARGETED DELIVERY OF SHORT HAIRPIN RNA EXPRESSING PLASMID USING HEPATITIS B VIRUS-LIKE PARTICLE FOR *Bcl-2* GENE SILENCING IN CERVICAL CANCER CELLS

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October 2021

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Faculty : Biotechnology and Biomolecular Sciences

Gene therapy research has advanced rapidly to clinical trials, but it is greatly hampered by the unstable nucleic acids particularly short interference RNA (siRNA) packaged inside carriers, and the lack of specificity towards targeted sites in the body. Hence, development of a stable carrier with specific targeted delivery is urgently needed. This study aimed to address gene therapy limitations by encapsidating a plasmid synthesizing short hairpin RNA (shRNA) that targets the anti-apoptotic *Bcl-2* gene (namely PshRNA) using truncated hepatitis B virus core antigen (tHBcAg) virus-like particle (VLP). A siRNA sequence targeting the anti-apoptotic *Bcl-2* was synthesized and cloned into pSilencer 2.0-U6 vector, and encapsidated inside tHBcAg VLP. The VLP encapsidating PsiRNA was conjugated with folic acid (FA) to produce FA-tHBcAg-PsiRNA VLP. Scanning transmission electron microscopy revealed that FA-tHBcAg-PsiRNA VLP has icosahedral structure similar to that of the unmodified tHBcAg VLP. Delivery of FA-tHBcAg-PsiRNA VLP into HeLa cells overexpressing folate receptor (FR) significantly downregulated the expression of anti-apoptotic *Bcl-2* at 48- and 72-hours post-transfection. MTT assay demonstrated that the cells' viability was significantly reduced from 89.46% at 24 h to 64.52% and 60.63%, respectively, at 48- and 72-hours post-transfection. As a conclusion, tHBcAg VLP can be used as a carrier for a receptor-mediated targeted delivery of a therapeutic plasmid encoding shRNA for gene silencing in cancer cells.

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

PENGHANTARAN SASARAN PLASMID MENGEKSPRESI RNA PENGGANGGU PENDEK DENGAN MENGGUNAKAN PARTIKEL MENYERUPAI VIRUS HEPATITIS B UNTUK MELENYAPKAN GEN *Bcl-2* DALAM SEL KANSER SERVIKS

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Penyelidikan terapi gen telah maju dengan cepat ke peringkat ujian klinikal, tetapi sangat terhambat oleh asid nukleik yang tidak stabil terutama “RNA pengganggu pendek” (siRNA) yang dibungkus di dalam pembawa, dan kekurangan pengkhususan terhadap lokasi yang disasarkan di dalam badan. Oleh itu, pembangunan pembawa yang stabil dengan penyampaian sasaran tertentu sangat diperlukan. Kajian ini bertujuan untuk mengatasi batasan terapi gen dengan membungkus plasmid yang mensintesis “RNA pin rambut pendek” (shRNA) yang mensasarkan gen *Bcl-2* anti-apoptosis (PshRNA) dengan menggunakan partikel menyerupai virus (VLP) antigen teras virus hepatitis B terpangkas (tHBcAg VLP). Jujukan siRNA yang mensasarkan Bcl-2 anti-apoptosis disintesis dan diklon ke dalam vektor pSilencer 2.0-U6, dan dibungkus di dalam tHBcAg VLP. VLP mengandungi PsiRNA dikonjugasi dengan asid folik (FA) untuk menghasilkan FA-tHBcAg-PsiRNA VLP. Mikroskopi elektron menunjukkan bahawa FA-tHBcAg-PsiRNA VLP mempunyai struktur icosahedral yang serupa dengan tHBcAg VLP asal. Penghantaran FA-tHBcAg-PsiRNA VLP ke dalam sel HeLa yang terlalu banyak mengekspresikan reseptor folat (FR), merencat pengekspresan anti-apoptosis *Bcl-2* pada 48 dan 72 jam selepas transfeksi. Ujian MTT menunjukkan bahawa keboleh hidupan sel berkurangan dengan ketara dari 89.46% pada 24 jam kepada 64.52% dan 60.63%, masing-masing, pada 48 dan 72 jam selepas transfeksi. Sebagai kesimpulan, tHBcAg VLP dapat digunakan sebagai pembawa untuk penyampaian yang disasarkan oleh reseptor untuk plasmid terapeutik yang mengekod shRNA untuk merencat gen dalam sel barah.

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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

BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bcl-2	*B cell lymphoma 2*
BSA	Bovine serum albumin
C-terminus	Carboxy terminus
CBB	Coomassie brilliant blue
Da	Dalton
dH2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dsRNA	Double stranded RNA
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylene di-amine tetra-acetic acid
FA	Folic acid
FA-tHBcAg	tHBcAg conjugated with folic acid
FA-tHBcAg-PshRNA	tHBcAg encapsidating plasmid expressing shRNA conjugated with folic acid
FBS	Fetal bovine serum
FR	Folate receptor
GFP	Green fluorescent protein
HBcAg	Hepatits B virus core antigen
HBV	Hepatitis B virus
HCl	Hydrochloric acid
IPTG	Isopropyl-β-D-thiogalactopyranoside

LB	Luria Bertani
M	Molar
Mg	Milligram
mL	Milliliter
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PshRNA	Plasmid DNA expressing shRNA
RNA	Ribonucleic acid
RNase	Ribonuclease
RPM	Rotation per minute
SDS	Sodium dodecyl sulphate
shRNA	Short hairpin RNA
siRNA	Short interference RNA
STEM	Scanning transmission electron microscopy
Sulfo-NHS	N-hydroxysulfosuccinimide
T	Triangulation number
TBS	Tris-buffered saline
tHBcAg	Truncated hepatitis B virus core antigen
tHBcAg-PshRNA	tHBcAg encapsidating plasmid expressing shRNA
UV	Ultra violet
VLP	Virus-like particle
VLPs	Virus-like particles

w/v	Weight over volume
µg	Microgram
µL	Microliter

CHAPTER 1

INTRODUCTION

It is estimated that there are 19.3 million new cases and 10 million deaths of cancers in 2020 (Sung et al., 2021). In this era, the treatments available for cancers are surgery, chemotherapy, radiation, immunotherapy, targeted drug therapy, and others. The choice of treatments depends on the location and severity of the cancers. Cancer treatments have some limitations, for instance surgery could not completely remove tumor in the unresectable area which leads to recurrence of the cancer if the cancer reaches metastasis. Besides, the side effects of chemotherapy and radiation can dramatically decrease the quality of patients' lives (Arbyn et al., 2020; Bray et al., 2018). One of the cancer treatments available in clinical trial is gene therapy.

Gene therapy study has advanced rapidly in the past decade and it has reached to clinical trials (Amer, 2014). Generally, gene therapy functions by either replacing faulty genes with functioning genes, suppressing the expression of harmful genes by using short interference RNA (siRNA), or introducing a new gene to prevent or help fighting disease with plasmid DNA vaccine (Ginn et al., 2018; Naldini, 2015). The present study employed a plasmid DNA expressing a short hairpin RNA (shRNA) inside cancer cells. The shRNA is transcribed by RNA pol III in the nucleus, and transferred to the cytoplasm where a dicer converts the shRNA into siRNA and forms RNA-induced silencing complex (RISC), which binds and cleaves the target mRNA (Kunkel & Pederson, 1989; Sliva & Schnierle, 2010; Zhou et al., 2014). However, the delivery of siRNA and the plasmid DNA into the cells remains a major challenge in clinical trials due to their limitations such as poor cellular uptake and easily excreted by kidney (Lowe et al., 2006; Zhou et al., 2014). An ideal delivery system should facilitate endosomal or lysosomal escape, have high transfection efficiency, specificity, and low toxicity (Burnett et al., 2011; Sliva & Schnierle, 2010; Zhou et al., 2014).

To date, viral vectors such as adenovirus and retrovirus are used to deliver shRNA, but these viral vectors have some toxic effects on transfected cells (Burnett et al., 2011; Sliva & Schnierle, 2010). Hence, nanoparticle-based delivery with high transfection, specificity, and low toxicity has been extensively studied to address the limitations of gene delivery (Al-Dosari & Gao, 2009; Burnett et al., 2011). The most commonly used nanoparticles are liposomal-, polymer-, metal- and protein-based nanoparticles (Rohovie et al., 2017). While the liposomal-based nanoparticles have been approved for delivery of a cancer drug, doxorubicin (Liu et al., 2017; Malam et al., 2009), these nanoparticles are less stable, rapidly removed by the immune system, and have unspecific delivery to healthy cells due to spontaneous membrane fusion (Allen & Cullis, 2013; Pattni et al., 2015). The polymer-based nanoparticles do not have uniform shapes. They are immunogenic and less stable (Elsabahy & Wooley, 2012).

However, the metal-based nanoparticles pose toxic effects to cells and lack of target specificity (Wang et al., 2012). Therefore, tremendous efforts have been made to produce more stable, homogeneous, less toxic, less immunogenic and highly specific nanoparticles. Among these nanoparticles, virus-like particles (VLPs) fulfill most of the criteria to be further developed as drug and gene delivery nano-vehicles.

VLPs are made of viral proteins, of which the particles formed and mimicked the conformation of authentic native viruses, but they lack the viral genomes (Roldão et al., 2010). One of the most extensively studied VLPs is the hepatitis B core antigen (HBcAg) VLP. With respect to HBcAg VLPs, truncated HBcAg (tHBcAg) without the arginine rich region has been studied by many researches (Tan et al., 2003; Tang et al., 2007). The tHBcAg VLP has been studied to encapsidate and deliver anti-cancer drugs, green fluorescent protein, fluorescein and oligonucleotide to mammalian cells (Biabanikhankahdani et al., 2016, 2017, 2018; Gan et al., 2018; Lee et al., 2012; Lee & Tan, 2008). However, the potential of tHBcAg as a plasmid DNA carrier has not been studied yet. In the present study, tHBcAg VLP was used to package a plasmid (3.2 kbp) expressing a shRNA that targets *B cell lymphoma-2* gene (*Bcl-2*) to study the potential of tHBcAg as nanocarrier for plasmid DNA.

In order to improve the targeting specificity of tHBcAg VLP, folic acid (FA) was conjugated to the primary amine groups exposed on the surface of the particle using EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide) and sulfo-NHS (*N*-hydroxysulfosuccinimide) coupling method. As FA interacts specifically with the folate receptor (FR), which is highly expressed in many cancer cells, including cervical cancer cells (Siwowska et al., 2017), tHBcAg VLP encapsidating the plasmid expressing shRNA (PshRNA) was conjugated with FA (FA-tHBcAg-PshRNA) to transfect HeLa cells (Figure 1.1).

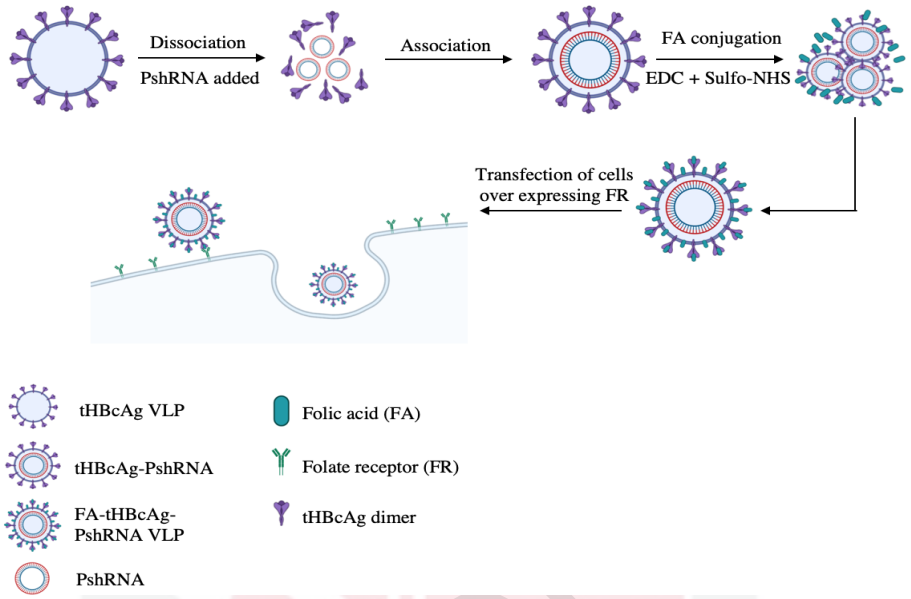

Figure 1.1 : Specific delivery of shRNA vectors to cells overexpressing folate receptor using hepatitis B truncated core antigen virus-like particles. Hepatitis B truncated core antigen (tHBcAg) VLPs are dissociated into tHBcAg dimers using urea. Plasmid expressing shRNA (PshRNA) is added to the dissociated tHBcAg dimers, and the urea is removed to re-associate the tHBcAg VLPs and encapsidate PshRNA, in which the tHBcAg-PshRNA VLPs are formed. The tHBcAg-PshRNA VLPs are then conjugated with folic acid (FA) using the EDC and sulfo-NHS coupling method to produce FA-tHBcAg-PshRNA VLPs, which are then used to deliver PshRNA to cells overexpressing folate receptor (FR)

It is hypothesized that tHBcAg VLP is an effective carrier to deliver plasmid DNA expressing shRNA into the targeted cancer cells (HeLa) for cancer treatment. Hence, the objectives of this study were:

1. To construct plasmid DNA which express shRNA in mammalian cells.
2. To encapsidate the plasmid DNA expressing shRNA inside tHBcAg VLP.
3. To conjugate FA to the surface of tHBcAg VLP.
4. To determine the morphology of tHBcAg VLP encapsidating plasmid DNA expressing shRNA and conjugated with FA.
5. To deliver the plasmid DNA expressing shRNA using tHBcAg VLP conjugated with FA to HeLa cells.
6. To determine the effect of plasmid DNA expressing shRNA towards HeLa cells.

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