PRELIMINARY STUDY ON PORPHYRIN DERIVATIVES AS TRANSFECTION REAGENTS FOR MAMMALIAN CELL

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Dedicated to:

My beloved mother and father

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ABSTRACT

Porphyrins are organic, aromatic compounds found in heme, cytochrome, cobalamin, chlorophyll and many other natural products with essential roles in biological processes that their cationic forms has been used as a groups of favorable non-viral vectors recently. Cationic porphyrins are self-chromogenic reagents with high capacity for modifications, great interaction with DNA and protection of DNA from nuclease during delivery of it into cell with low toxicity. In order to have high efficient gene transfection into cell while causing low toxicity, genetically manipulations of non-viral vector, cationic porphyrin, would be useful. In this study newly modified cationic porphyrins namely, 5-hexyl-10,15,20tris (*N*-methyl-4-pyridyl) porphyrin, 5-propyl-10,15,20tris (*N*-methyl-4-pyridyl) porphyrin, 5,10-dipropyl-15,20-bis (*N*-methyl-4-pyridyl) porphyrin, 5,10-dihexyl-15,20bis (*N*-methyl-4-pyridyl) porphyrin, and polyamidoamine (PAMAM) G_4-porphyrin conjugate were applied. Cytotoxicity of synthesize cationic porphyrins on Chinese Hamster Ovarian (CHO) cells, were evaluated by using MTT assay. Generally, all cationic derivatives are dose dependent, with low cytotoxicity at the ranges from 100 µM to 0.01µM. Four of cationic porphyrin were uptake by cell at high concentration while none were observed on conjugate one. Using different concentration of cationic porphyrins and methods were tested on transfection of CHO cells by using the derived transfection reagent with X-tremeGENE HP DNA as positive control. However no transfection observed by all the porphyrin derivatives and the parameters tested except for positive control. Results of this study suggested that applying different protocol, and also trying other concentration of cationic porphyrins and DNA for forming a strong complex would increase the possibility of efficient gene transfection by using cationic porphyrins.

ABSTRAK

Porphyrin ialah sebatian aromatic organik yang boleh ditemui di dalam hem, sitokrom, kobalamin, klorofil dan pelbagai lagi produk semulajadi. Dengan ciri kation, ia mempunyai fungsi yang penting dalam proses biologi semulajadi dan boleh dimanipulasi sebagai vector bukan viral dalam proses transfeksi. Porphyrins kation ialah bahan kimia kromogenik kendiri yang mempunyai cirri-ciri seperti kapasiti yang tinggi untuk diubahsuai, interaksi yang baik dengan DNA dan dapat melindungi DNA daripada nuklease semasa pemindahannya kedalam sel dibawah toksisiti yang rendah terhadap sel. Untuk mendapatkan transfeksi gen yang cekap kedalam sel dengan toksisiti yang rendah serta vektor bukan virus untuk pengubahsuai genetic, kationik porphyrin mungkin berguna untuk tujuan ini. Dalam kajian ini, kationik porphyrins yang digunakan ialah 5-hexyl-10,15,20tris (*N*-methyl-4-pyridyl) porphyrin, 5-propyl-10,15,20tris (*N*-methyl-4-pyridyl) porphyrin, 5,10-dipropyl-15,20-bis (*N*-methyl-4-pyridyl) porphyrin, 5,10-dihexyl-15,20bis (*N*-methyl-4-pyridyl) porphyrin, dan konjugat polyamidoamine (PAMAM) G_4-porphyrin. Kajian MTT digunakan untuk menentukan sitotoksisiti kationik porphyrins keatas Sel ovari hamster China (CHO). Secara umunya, kesemua terbitan kationik berkadar terus dengan dose toksisiti yang rendah dalam julat 100 µM to 0.01µM. Empat daripada kationik porphyrin telah diambil oleh sel pada kepekatan yang tinggi dan tidak pada sebatian konjugat. Dengan menggunakan kationik porphyrin pada kepekatan yang berbeza dan kaedah tranfeksi keatas sel CHO dan X-tremeGENE HP DNA telah digunakan sebagai kawalan positif. Walau bagaimanapun tiada transfeksi didapati oleh semua terbitan porphyrin dan parameter yang diuji kecuali kawalan positif. Keputusan kajian ini mencadangkan bahawa menggunakan protokol yang berbeza, dan juga julat kepekatan porphyrins kationik dan DNA yang lain untuk membentuk sebuah kompleks yang kukuh akan meningkatkan kemungkinan penggunaan porphyrins kationik sebagai transfeksi gen yang cekap.

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LIST OF ABBREVIATIONS

µg	-	Microgram
µM	-	Micro Molar
µL	-	Micro Litter
ANOVA	-	Analysis Of Variance
cDNA	-	complementary deoxyribonucleic acid
CHO	-	Chinese Hamster Ovary
CO2	-	carbon dioxide gas
CTAB	-	cetyltrimethylammonium bromide
dH_2O	-	distilled water
DMSO	-	Dimethyl sulfoxide
DMEM	-	Dulbecco's Modified Eagle Medium
DNA	-	deoxyribonucleic acid
DNase	-	deoxyribonuclease
EGFP	-	Enhanced green Fluorescent protein
ELISA	-	Enzyme-linked immunosorbent assay
FBS	-	fetal bovine serum
G	-	Gram
GFP	-	Green fluorescent protein
HCl	-	hydrochloric acid
LB	-	Luria Broth
min	-	minute(s)
ml	-	Mili Litter

MTT assay		3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	-	molecular weight
NaCl	-	sodium chloride
NaOH	-	sodium hydroxide
nm	-	nanometer
Pcmv	-	Cephalomyocarditis virus plasmid
PBS	-	Phosphate Buffer Saline
PEI	-	Polyetheleneamine
PAMAM	-	polyamidoamine G4-porphyrin conjugate
Pophyrin.D1	-	5-hexyl-10,15,20tris(N-methyl-4-pyridyl)porphyrin
Pophyrin.D2	-	5-propyl-10,15,20tris(N-methyl-4-pyridyl)porphyrin
Pophyrin.D3	-	5,10-dipropyl-15,20-bis (N-methyl-4-pyridyl)porphyrin,
Pophyrin.D4	-	5,10-dihexyl-15,20bis(N-methyl-4-pyridyl)porphyrin
RNA	-	ribonucleic acid
RNase	-	ribonuclease
rpm	-	round per minute
SDS	-	Sodium dodecyl sulfate
Sec	-	second(s)
UV	-	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Introduction

Gene transfection is a technique aiming to carry genetic material and introduce them to mammalian cells. This process is done by physical and chemical methods to study about gene function, protein expression and therapeutic aims.

Gene therapy is a new method to improve genetic disorder. These diseases are caused because of malfunction of molecules at cellular level. Centre for Genetics Education defines this method as a way of treatment which has been started practically in 1990. It aims to deliver a healthy or modified gene into cell in order to act as a new gene, correct it, or to replace a defective gene. Expression of therapeutic gene result in medical effects. In spite of being a useful method of curing diseases, only limited successes have been achieved up until now. Several factors such as having enough capacity for carrying great genes, safe and easy method of use, capacity of different modification, being high efficient, less immune response in body, economical, high stability, high biodegradability, low toxicity, and ability to target to exact part of the cell are limitation for the success (Huang *et al.*, 2005). Consequently, delivery of oligonucleotides and other macromolecules into cells must be done by appropriate reagent and applying special methods to have a successful gene delivery. Generally, vectors for gene delivery are divided in two groups of viral

and non-viral ones, in addition to physical methods. These terms will be comprehensively discussed later in literature review.

1.2 Research Background

Gene delivery refers to use of viral or non-viral vectors as a vehicle or applying physical methods to transfer genetic material into the cells.

Viral vectors are replication-defective viruses. They contains coding sequence of virus which is replaced by that of therapeutic genes (Huang *et al.,* 2005).They have two critical factors for an efficient gene delivery. One factor is their exclusive performance to transfer gene into target cells of specific tissue. Another factor is carrying that therapeutic DNA into the desired cell's nucleus. Viral vectors have natural system of transferring genes. It makes them able to attach to the cell, pass barriers of it and finally become uptake by nucleus (Ragusa *et al.,* 2007). These characteristics provide an efficient gene delivery performed by engineered viruses.

Various modified viruses are applied as gene vector like, Retroviruses, Adenoviruses, Adeno-associated viruses and other kinds of viruses. Retroviruses are RNA viruses with the ability of causing long-term gene expression if used as a viral vector. However there is a risk of causing insertational mutagenesis (Kay *et al.,* 2001). Adenoviruses with DNA are another kind of viral vectors. They can be applied for non-dividing cells as well as dividing ones. However they may cause short-term gene expression or lead to immune response in body (Ragusa *et al.,* 2007). Based on Promega Protocols & Applications Guide, they can be modified to be used for carrying large DNAs. Adeno-Associated viruses need aid of helper viruses and are applied for both kinds of cells, divided and non-divided ones. On the other hand they cannot carry great genes (Huang *et al.,* 2005). Even though viral

reagents are good options for gene therapy because of leading to high efficient *in vivo* gene delivery, there is a risk of causing insertational mutagenesis, replication-induced infections, and immunogenicity.

Non-viral transfection methods and reagents can be classified in groups namely, physical methods ,cationic lipids, cationic polymers, complex of them, and porphyrins.

There are several physical methods for gene transfection. Gene gun or particle bombardment is one of them in which DNA will be shoot into the cells. In gene electroporation, another physical method, electrical pulses leads to formation of pores in membrane of cell. Pores lets DNA passes through them into the cell. Direct microinjection using needle is another costly physical method of gene transfection. Most important disadvantage of using physical methods is that they may cause cell membrane defects and cell death.

Non-viral reagents are another option for gene delivery. Several advantages of using these vectors make them a prior choice. Besides being safe, recent development in manipulation of transfection reagents such as cationic lipids, cationic polymers, cationic porphyrins, increase usage of them as the first option for gene therapy. Depend on the type of the cell, transfection reagents have slow entrance into the cells. They coat DNA or provide positive charges or some time neutralize charge of DNA which is naturally, negatively charged due to phosphate backbones.

Cationic lipids are made of three important parts namely, polar head to interact with DNA, lipid chain for formation of liposome, and linker for linking mentioned parts together. Formation of lipoplex consist of DNA and cationic lipid ends to gene transfection.

Cationic polymers are arranged in two groups of natural and synthetic. polysaccharides, proteins, and peptides are examples of natural cationic polymers and dendrimers, polyphosphosters are two of most important examples of synthetic ones (Ragusa *et al.,* 2007). These vectors form polyplex by DNA via their amines or ammonium ions that leads to electrostatic interaction between them. They are known as chemical reagent as well. Capability of being used for all cell types and having a simple practical protocol increase use of them for therapeutic aims.

Complex of cationic polymers and lipids by DNA called lipopolyplex which saves DNA from degradation by nuclease enzymes (Ragusa *et al.,* 2007).

Porphyrins are organic, aromatic compounds that has been recently used as novel gene transfection reagents. There are two groups of them. One group is metal complex porphyrins and another group is metal free complex porphyrins. Both groups have a great capacity of acceptance of many modifications, carrying macromolecules into cells and protecting them from DNA nuclease enzyme while causing low level of toxicity. Due to being a part of many natural compounds like haemoglobin, it is believed that they cause no immune response in human body and have high biocompatibility with cells. Their important roles as gene transfection reagents for gene therapy studies such as curing cancer is seen in many studies.

As many studies show, non-viral vectors are more advantageous for gene therapy in comparison with viral ones. It is because these vectors are less expensive to work with, carrying great genes, cause less immune response, easier and safer method of using, have specificity for gene delivery (Fortune *et al.,* 2011), high stability (Ahn *et al.,* 2008) and also the ability for numerous chemical modifications. Polyethylenimine (PEI) which is used widely as a cationic polymer transfection reagent was successfully used for gene delivery into the rodent brain. It led to high expression of desired protein which ended to wanted phenotypic changes (Mahmoodi *et al.*, 2004). In another study, transfection of HUVEC (Human Umbilical Vein Endothelial Cells) was done successfully by using chemical transfection reagents

(Hunt *et al.*, 2010). Some porphyrin derivatives have ability to not only deliver the oligonucleotides into mammalian cell by saving it from nuclease degradation, but also increase its uptake by target cells. However there are some limitations for using them *in vivo* like low biodegradability which leads to accumulation of toxic material in cell and low efficient gene transfection (Patnaik *et al.*, 2011).

1.3 Problem Statement

To have a successful gene therapy, a vector should have favorable characteristics that guarantee high efficient gene delivery. Chosen vector must be able to interact properly with DNA and be able to carry it into cell. It should pass all obstacles and barriers of cell and be able to overcome negative charges of cell membrane and interact with it. Furthermore, it should has characteristics that makes it preferable compared to other vectors and methods. Viral vectors have been applied widely for therapeutic aims however they have risk of causing immune response in human body. On the other hand, many non-viral vectors have the possibility of leading to toxicity if being used at high concentration. In this study to solve these problems, synthesized cationic porphyrin compounds' derivatives were used as non-viral gene transfection reagents. It is believed that they have the potential of being used as a suitable vector to interact with DNA efficiently, pass physiological barriers and extra matrix, cause low level of toxicity and highest gene transfection efficiency.

1.4 Objective of Study

I. To evaluate cytotoxicity of cationic porphyrin derivatives on cultured CHO cells by applying MTT assay.
II. To establish cellular uptake of cationic porphyrin derivatives in CHO cells.
III. To transfect CHO cell by using non-toxic concentrations of cationic porphyrin derivatives.

1. 5 Scope of Study

This study focuses on gene transfection into Chinese Hamster Ovarian (CHO) cells by using porphyrin compounds' derivatives as vectors. To achieve this goal, cytotoxicity of porphyrin derivatives on CHO cells were evaluated at the ranges of 0.01 µM to 1000 µM. Different concentrations of cationic porphyrins were tested on cellular uptake and transfection of CHO cells by using the derived transfection reagents namely, 5-hexyl-10,15,20tris (*N*-methyl-4-pyridyl) porphyrin, 5-propyl-10,15, 20tris (*N*-methyl-4-pyridyl) porphyrin, 5,10-dipropyl-15,20-bis (*N*-methyl-4-pyridyl) porphyrin, 5,10-dihexyl-15,20bis (*N*-methyl-4-pyridyl) porphyrin, and polyamidoamine (PAMAM) G_4-porphyrin conjugate with structures that are shown in the next page, in Figure 1.1.

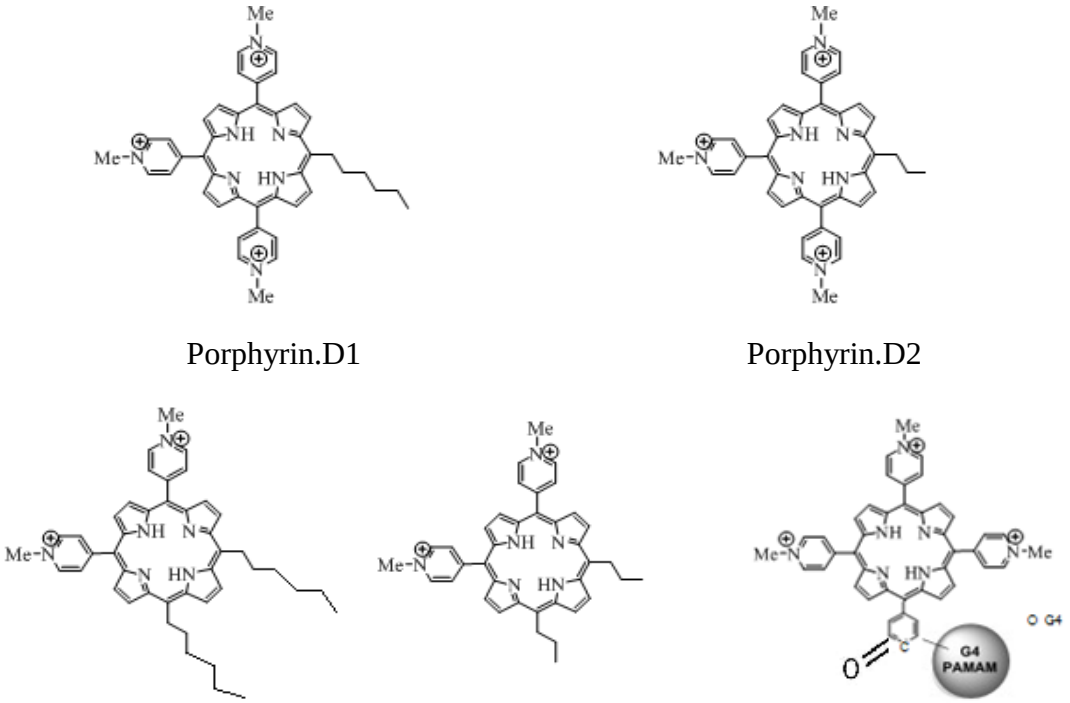

Figure 1.1 Cationic porphyrin derivatives' structures (Kiew Siaw Fui, Department of Chemistry, Faculty of Science, UTM, 2012)

1.6 Significant of Study

It is hypothesized that newly synthesized cationic porphyrins namely, 5-hexyl-10,15,20tris (*N*-methyl-4-pyridyl) porphyrin, 5-propyl-10,15, 20tris (*N*-methyl-4-pyridyl) porphyrin, 5,10-dipropyl-15,20-bis (*N*-methyl-4-pyridyl) porphyrin, 5,10-dihexyl-15,20bis (*N*-methyl-4-pyridyl) porphyrin, and polyamidoamine (PAMAM) G_4-porphyrin conjugate have the potential of being applied as suitable vectors to carry EGFP plasmid into CHO cells. This study can be used later as a model to study other type of cells and pave ground to treatment of disease by gene therapy.

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