UNIVERSITI PUTRA MALAYSIA

SYNTHESIS AND CHARACTERIZATION OF NOVEL PLASMID-LAYERED DOUBLE HYDROXIDES NANOBIOHYBRIDS FOR GENE DELIVERY

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Dedicated to arwah Atuk Yusof, and arwah Wan Zaharah.

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

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By

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October 2008

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Faculty : **Biotechnology and Biomolecular Sciences**

One of the hindering problems faced by conventional gene delivery systems into cells is their efficiency in its delivery and integration. DNA and other genetic materials are easily degraded in both the extracellular as well as intracellular matrix by both endonuclease activities and physiological conditions of the cellular environment. Therefore, research insights have focused on utilizing the emerging field of nanotechnology to overcome this problem. For this reason, a layered nanomaterial, Mg/Al-LDH based on the layered double hydroxide (LDH) system was synthesized at pH 10.0 at different Mg to Al ratios, to determine whether it can be used as a vector for gene delivery. A plasmid DNA, encoding the green fluorescent protein reporter gene, was intercalated into the LDH intergallery region; previously occupied by nitrate anions. Successful occupation of the

circular DNA was confirmed by expansions within the intergallery spacing of the LDH from powder x-ray diffraction analysis. Fourier-transform infrared spectroscopy further revealed the presence of exclusive functional groups belonging to both DNA and LDH in the nanobiohybrid product, and by both CHNS as well as gel electrophoresis data, the plasmid DNA was confirmed to be successfully intercalated within the LDH host. The effects of the host on cells were then evaluated using MTT assay on two cell lines, and the synthesized LDH hosts were found to have no significant lethal effects on cell viability. Microscopic studies using SEM and TEM later revealed the nanobiohybrid size to be within the nano-meter range, which was found to enhance its uptake by cells. Cells transfected with the nanobiohybrid showed successful expression of the GFP gene compared to controls, as observed using fluorescence microscopy. The nanobiohybrid was found to not only deliver the gene into cells, but gene expression efficiency using the host for transfection was even comparable to a commercially available, high cost transfection vector in the market. Compared to the commercial vector, the LDH host was also shown to provide sufficient protection of the intercalated plasmid from degradation by the DNase I and *Xho*I/*Kpn*I restriction enzymes, showing the potential of using the LDH host as an alternative delivery vector for gene delivery.

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

SINTESIS DAN PENCIRIAN NANOBIOHIBRID PLASMID-LAPISAN BERGANDA HIDROKSIA UNTUK PEMINDAHAN GEN

Oleh

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Oktober 2008

Pengerusi : Professor Datin Khatijah Mohd. Yusoff, PhD

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Salah satu dari masalah yang sering dihadapi berkenaan proses transfeksi gen ke dalam sel adalah dalam pemindahan serta integrasinya ke dalam genom sel perumah. Ini kerana, DNA serta bahan genetik seumpamanya amat mudah mengalami degradasi di persekitaran dalam dan luar sel; melalui tindakan enzim-enzim *endonuclease*, di samping keadaan fisiologi sel terbabit. Oleh itu, untuk menangani masalah ini, penyelidikan dalam terapi gen telah melibatkan penggunaan sains nanoteknologi, yang umumnya semakin mendapat perhatian. Di dalam penyelidikan ini, sejenis bahan nano yang berasaskan kepada sebatian lapisan berganda hidroksida (LDH), Mg/Al-LDH telah disintesis pada pH 10 dalam komposisi Mg dan Al yang berbeza, bagi menilai keupayaan bahan nano ini untuk dibangunkan sebagai suatu vektor alternatif bagi transfeksi gen. Satu plasmid DNA, mengandungi gen bagi expresi protein floresen hijau dalam sel, telah diinterkalasikan di antara ruangan lapisan LDH tersebut. Dengan

menggunakan teknik pembelauan sinar x-ray, pembentukan sebatian nanohibrid baru yang mengandungi DNA plasmid diinterkalasikan ke dalam LDH telah ditentukan; melalui peningkatan saiz ruang antara lapisan LDH tersebut yang lebih besar. Daripada spektrum F-TIR yang diperoleh, sampel nanobiohibrid yang telah disintesis itu didapati mengandungi struktur kumpulan-kumpulan kimia yang dipunyai kedua-dua DNA plasmid dan LDH. Analisis kandungan menggunakan teknik CHNS serta elemen organik-tak organik, menunjukkan bahawa sampel nanobiohibrid tersebut mempunyai komponen DNA, dan seterusnya membuktikan bahawa interkalasi DNA plasmid telahpun berlaku. Analysis ketoksikan menggunakan asei MTT telah dijalankan bagi menentukan sama ada bahan nano LDH tersebut adalah toksik terhadap sel-sel yang ingin ditransfeksi. Asei tersebut mendapati bahawa bahan nano LDH yang digunakan adalah tidak toksik kepada sel. Analisis mikroskopi SEM dan TEM mendapati semua sampel LDH dan nanobiohybrid tersebut berbentuk heksagon serta wujud pada saiz nanometer. Ekspresi gen dalam sel dengan transfeksi menggunakan sampel nanohibrid yang telah disintesis mendapati bahawa bahan nano LDH tersebut mampu memindahkan gen GFP ke dalam sel. Menggunakan mikroskop floresen, kadar expresi gen positif menggunakan bahan nano tersebut adalah tinggi, dan setaraf berbanding transfeksi menggunakan suatu vektor transfeksi gen komersial, tetapi didapati pada harga yang lebih tinggi. Dengan menggunakan bahan nano tersebut sebagai vektor transfeksi, didapati bukan sahaja boleh memindahkan gen tersebut dengan baik ke dalam sel, tetapi juga berupaya melindungi DNA plasmid yang diselitkan daripada tindakan enzim

pembatas *Xho*I/*Kpn*I serta enzim DNase I. Keupayaan bahan nano LDH ini dalam melindungi dan membantu meningkatkan pemindahan suatu gen ke dalam sel menunjukan keupayaan dan potensi untuk digunakan serta dibangunkan sebagai suatu vektor transfeksi alternatif untuk teknologi pemindahan gen.

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I certify that an Examination Committee met on 10th October 2008 to conduct the final examination of Mas Jaffri Masarudin on his Master of Science entitled "Synthesis and Characterization of Novel Plasmid-Layered Double Hydroxides Nanobiohybrids for Gene Delivery" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universitti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the student be awarded the Master of Science.

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DECLARATION

I declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously and is not concurrently submitted for any other degree at UPM or at any other institution.

MAS JAFFRI MASARUDIN

Date: 28 October 2008

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LIST OF ABBREVIATIONS

Å	Angstrom
ADA	Adenosine deaminase
ATP	Adenosine triphosphate
NB	Nanobiohybrid(s)
CHO	Chinese Hamster Ovarian
C	Carbon
CHNS	Carbon, Hydrogen, Nitrogen, Sulphur
CNT	Carbon nanotubes
CO_2	Carbon dioxide
°C	Degree Celsius
CMP	Cytosine Mono Phosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
FCS	Foetal Calf Serum
F-TIR	Fourier-Transform Infrared
GFP	Green Fluorescent Protein
µg	microgram
mg	milligram
g	gram
h	hour
IC_{50}	Inhibition Concentration at 50% Viability

ICP-AES	Inductive Couple Plasma – Atomic Emission Spectroscopy
LDH	Layered Double Hydroxide
ml	millilitre
M	molar
M^{2+}/M^{II}	Divalent metal cation
M^{3+}/M^{III}	Trivalent metal cation
mRNA	Messenger RNA
MTT	methyl thiazol tetrazolium bromide
MTX	Methotrexate
N	Nitrogen
nm	nanometer
PBS	Phosphate Buffered Saline
PEG	Poly Ethylene Glycol
PX-RD	Powder X-Ray Diffraction
RNA	Ribonucleic Acid
RNAi	RNA interference
SEM	Scanning Electron Microcope
siRNA	Small interfering RNA
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
TGA-DTG	Thermal Gravimetry Analysis – Derivatives Thermal Gravimetry
Θ	X-Ray diffraction angle

CHAPTER 1

INTRODUCTION

Many recent understandings and significant conceptual findings of modern day research have evolved to include a mainly interdisciplinary approach; slowly diminishing the fine line separating the many fields of physical, chemical and biological sciences. These revolutions in how researchers perceive, conceive, and conduct their work are now inclined towards the early conceptual genesis of nanobiotechnology - the fusion between biotechnology; the field of biological sciences directed at the manipulation of biological systems, with nanotechnology; the science of materials one-billionth of a metre.

The emphasis over nanobiotechnological research throughout the last few years, has been tremendously directed on the fabrication of various nanomaterials (Jain, 2005) such as inorganic and organic nanoparticles, fullerenes, dendrimers and layered double hydroxides for delivery of therapeutics; well-designed to suit and overcome the many biological barriers that presently hinders present drug delivery systems. Usually referred to as non-viral vectors, their usage has received considerable interest over the past few years. Using these materials is advantageous due to its ability to be designed and synthesized from the ground up for tailored specificity;

henceforth leading towards the discovery of new drug and delivery systems. Because of its very small size (~10^{-9} m) uptake into cells would be vastly improved by either simple diffusion or endocytosis, or even by enzymatic breakdowns in the acidic environments of the lysosome (Kwak *et al*., 2004).

The efficient delivery for new-age therapeutics such small-interfering RNA (siRNA), DNA and RNA aptamers as well as plasmid DNA for gene therapy faces many hindering biological barriers. Direct administration of such biomaterials would usually result in their premature denaturation and degradation within cellular environments (Xu *et al*., 2006). This is because both the intracellular as well as extracellular spaces of cells are flooded with the actions of endonucleases (enzymes) that cleave and degrade foreign invasive DNAs entering the cells. Due to these obstacles, therapeutic DNA relies solely upon the development of various delivery vector systems.

Layered double hydroxides (LDH) are anionic clays that have the ability to encapsulate, or 'contain' various negatively charged moieties within its interlayer spacing. The process, known as intercalation, involves the insertion of a guest material within a layered material to retain its compositional structure (Cavani *et al.,* 1991). This inorganic, nano-sized material can therefore be used to accommodate many bioactive materials, such as oligopeptides and amino acids (Aisawa *et al*., 2006), DNAs (Oh *et al*., 2006), pesticides (Li *et al*., 2004b), herbicides (Legrouri *et al*., 2005), as well as hydrophobic molecules (Wang *et al*., 2005) by electrostatic interactions within its intergallery spacing. Studies utilizing the nanomaterial have shown to

increase the delivery efficiencies of many biomolecules; which has included many drugs (Li *et al.,* 2004a; Oh *et al*., 2006a), antibiotics (Tammaro *et al.,* 2007; Trikeriotis and Ghanotaxis, 2007), and even therapeutic DNA and genes (Kwak *et al.,* 2002; Tyner *et al.,* 2004). Furthermore, encapsulation within an LDH host offers protection of the biomolecules from degradation by nucleases (Oh *et al.,* 2006a) and enzymes thus their delivery can also be dose-controlled.

This study aims to evaluate whether LDH can be used as an alternative gene delivery vector; by intercalating a type of circular DNA called plasmid DNA which contains a green fluorescent protein (GFP) gene into synthesized MgAl-NO_3^- LDH hosts. By using these inorganic hosts to encapsulate and protect the plasmid DNA within cellular environments, it is hypothesized that their uptake and expression in transfected cells will be enhanced and significantly increased compared to naked DNA delivery methods and conventional gene delivery vectors present in the market. Therefore the objectives set for this study are as follows;

1. to synthesize MgAl-NO_3^- layered double hydroxides via the coprecipitation method at different molar ratios (R_i),
2. to intercalate plasmid DNA into the synthesized MgAl-NO_3^-, and characterize the newly formed plasmid-LDH nanobiohybrids,
3. to evaluate any cytotoxicity the host, MgAl-NO_3^- has towards cells, and
4. to perform cellular transfection of cells using the synthesized nanobiohybrids for GFP gene expression.

CHAPTER 2

LITERATURE REVIEW

2.1 Nanotechnology for biological sciences

The term nanotechnology often conforms to many definitions in various modern sciences, but it has since been commonly regarded as the science of one-billionth of a metre. It involves the design, construction and use of functional nano-meter sized materials fabricated for a dimensional array of uses (Park *et al*., 2007b; Alexis *et al*., 2008). Although their sizes usually vary from 1-100 nm, by definition these small sizes must prove to play a vital part for its functionality.

The advent of nanotechnology has opened up many opportunities that can be utilized in a wide area of sciences such as engineering, material sciences, environmental sciences, healthcare and even the life sciences. However, research in the fields of nanomedicine and nanobiotechnology have been regarded to receive the most significant impact from this 'small-scaled' technology; with increasing interests into fabricating novel nanomaterials that have the ability to overcome many biological barriers currently present in biological systems (Alonso, 2004; Freitas Jr., 2005).

Such biological materials that are being developed are known as nanoparticles; spherical structures in the nanometer range sizes, extensively studied as a vector for improved delivery of functional biomaterials. Nanoparticles are highly flexible nano-structures due to their uniquely small sizes, which have been used to increase the delivery efficiency of various biomaterials. These materials are easily synthesized usually using polymeric biocompatible materials such as chitosan and dextran as the encapsulation vector; the particles are readily degraded within the cell upon entry to release the encapsulated biomaterials. Chen *et al.* (2007a) and Zhang *et al*. (2007) discovered in their studies that the encapsulation of an immunosuppressant drug, rapamycin and the diabetic drug, insulin within cholesterol and PEG-modified chitosan nanoparticles increased their permeability and delivery uptake into cells; showing a slow-sustained release of the proteins *in vivo*. While such properties were common in numerous other studies (Jiang *et al*., 2005; Wang *et al*., 2006; Krauland & Alonso, 2007; Puri *et al*., 2007), research has now progressed onto the development of new-targeted nanoparticles for specific delivery in the cell and human body (Jain, 2005; Rieux *et al*., 2006; Breunig *et al*., 2007).

Nanoparticles have been studied in the treatment of many types of cancer (Alexis *et al*., 2008) involving construction of novel nucleic acid-nanoparticle conjugates, which can target specific cancer genes for mRNA silencing (Woodle & Lu, 2005) for different cancers including human liver cancer (Park *et al*., 2007a) and breast cancer (Nguyen *et al*., 2007).