UNIVERSITI PUTRA MALAYSIA

SYNTHESIS AND *in vitro* VISUALIZATION OF FLUORESCEIN ISOTHIOCYANATE-LABELED CHITOSAN NANOPARTICLES (FITC-CNP) IN HUMAN KIDNEY CANCER CELLS

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By

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Thesis submitted to the Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Science
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SYNTHESIS AND *in vitro* VISUALIZATION OF FLUORESCEIN ISOTHIOCYANATE-LABELED CHITOSAN NANOPARTICLES (FITC-CNP) IN HUMAN KIDNEY CANCER CELLS

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The use of fluorescently-labeled nanoparticles has garnered attention in the medical field, due to the ability to track its uptake during cell transfection. In this study, the synthesis and *in vitro* visualization of fluorescein isothiocyanate (FITC)-labeled chitosan nanoparticles (CNPs) inside human kidney cancer cells (786-O cell line) were described. CNPs were prepared using a modified ionic gelation method to yield spherical nanoparticles, and fluorescent labeling of CNP was performed using N-hydroxy-succinimidyl (NHS)-FITC. Particle size distribution and polydispersity index of nanoparticles were analyzed using dynamic light scattering. Surface morphology was analyzed using FE-SEM. For transfection, 786-O human kidney cancer cells were established and subsequently treated with both CNP and FITC-CNP, visualized at three different time points using fluorescence microscopy. It was found that 600 µl of 0.5 mg/ml chitosan with 200 µl of 0.7 mg/ml TPP gave the optimum particle size and PDI value at 71.7$\pm$0.4 nm and 0.14$\pm$0.01, respectively. FE-SEM data obtained was consistent with DLS results at optimum parameters. Visualization of cell treatment showed that at 30 mins, all FITC-CNPs were seen as small green dots with weak fluorescence residing outside of the cells at close proximity to the cell membrane. Most

of the nanoparticles internalized into the cells at 6h were seen to be in the cytoplasm or surrounding the nucleus. As time progresses to 24h, stronger fluorescence was observed with all FITC-CNPs accumulating inside cells with smearing observed, probably due to FITC detachment from CNPs. These results implied that the synthesized FITC-labeled CNPs have the potential for use as carrier system for enhanced drug delivery into cells.

Keywords: FITC-labeled CNPs, 786-O cells, chitosan nanoparticles, drug delivery system, nanobiotechnology

Abstrak thesis yang dikemukan kepada Jabatan Biologi Sel dan Molekul
Sebagai syarat memenuhi keperluan untuk ijazah Sarjana Muda Sains (Kepujian)

SYNTHESIS AND *in vitro* VISUALIZATION OF FLUORESCEIN ISOTHIOCYANATE-LABELED CHITOSAN NANOPARTICLES (FITC-CNP) IN HUMAN KIDNEY CANCER CELLS

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Pengerusi: Dr. Mas Jaffri Masarudin, PhD
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Penggunaan partikel nano berpendafluor telah menarik perhatian para penyelidik terutamanya dalam bidang perubatan atas sebab kebolehannya untuk menjejaki pengambilannya ketika transfeksi sel. Dalam kajian ini, sintesis dan visualisasi *in vitro* bagi partikel nano kitosan (CNP) dilabel FITC (FITC-CNP) dalam sel kanser buah pinggang manusia (Titisan sel 786-O). Sintesis CNP disediakan dengan menggunakan kaedah penggelan ion yang diubah suai untuk menghasilkan partikel nano berbentuk sfera manakala pelabelan pendafluor kepada CNP dilakukan dengan menggunakan NHS-FITC. PSD dan PDI daripada partikel nano dianalisis menggunakan DLS manakala morfologi permukaan dianalisis menggunakan FE-SEM. Bagi transfeksi sel, titisan sel 786-O tetap dilaksanakan dan kemudiannya dirawat dengan CNP dan FITC-CNP, dan divisualisasi dalam tiga masa yang berbeza dengan menggunakan mikroskop pendafluor. Hasil kajian mendapati bahawa 600 µl 0.5 mg/ml chitosan dengan 200 µl of 0.7 mg/ml TPP memberikan saiz partikel yang optimum dan nilai PDI masing-masing pada 71.65 nm and 0.14. Data FE-SEM yang diperoleh adalah konsisten dengan keputusan DLS pada parameter optimum. Visualisasi untuk rawatan sel menunjukkan bahawa pada 30 min, semua FITC-CNP dilihat sebagai titik kecil hijau dengan

kependafluoran lemah yang berada di luar sel-sel tetapi berdekatan dengan membran sel. Kebanyakan partikel nano telah masuk ke dalam sel pada 6 j rawatan dan menetap sama ada dalam sitoplasma atau sekitar nukleus manakala setelah 24 j rawatan, kependafluoran yang lebih kuat diperhatikan dengan semua FITC-CNP yang terkumpul di dalam sel-sel dan calitan diperhatikan, mungkin disebabkan oleh penanggalan FITC daripada CNP. Hasil kajian yang didapati mengimplikasikan bahawa FITC-CNP yang disintesis mempunyai potensi untuk digunakan sebagai sistem pembawa ubatan yang efisien ke dalam sel-sel.

Kata Kunci: FITC-CNP, titisan sel 786-O, partikel nano kitosan, sistem pembawa ubatan

Approval

This thesis was submitted to the Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences and has been accepted as fulfillment of the requirement for the degree of Cell and Molecular Biology. The member of the Supervisory Committee was as follows:

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Declaration

Declaration by undergraduate student

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LIST OF ABBREVIATIONS AND SYMBOLS

A	-absorbance
^{o}	-degree
ɤ- Fe_2O_3	-superparamagnetic nanoparticles
C	-Celcius
CDDP	-cis-diamminedichloroplatinum (II)
CFM	-confocal fluorescence microscopy
CO_2	-carbon dioxide
CS	-chitosan
CNP/s	-chitosan nanoparticle/s
DLS	-dynamic light scattering
DMSO	-dimethyl sulfoxide
DNA	-deoxyribonucleic acid
DOX	-doxorubicin
EPR	-enhanced permeability effect
FE-SEM	-field emission-scanning electron microscopy
FITC	-fluorescein isothiocyanate
g	-grams
h	-hour(s)
HCl	-hydrochloric acid
HGC	-hydrophobically modified glycol chitosan

hMSCs	-human mesenchymal cells
HFT-T	-heparin-folic acid-paclitaxel loaded with paclitaxel
l	-litre
m	-metre
mg	-milligram
min	-minute(s)
ml	-mililitre
MNPs	-magnetic nanoparticles
MRI	-magnetic resonance imaging
MSNs	-mesoporous silica nanoparticles
NaOH	-sodium hydroxide
NaTC	-sodium taurocholate
nm	-nanometer
NPs	-nanoparticles
PBS	-phosphate buffer saline
PDI	-polydispersity index
PSD	-particle size distribution
rMSCs	-rat mesenchymal stem cells
rpm	-revolutions per minute
s	-second(s)
SiNPs	-silica nanoparticles

SiO_2	-silicon dioxide
TPP	-(sodium) tripolyphosphate
UV	-ultraviolet
%	-percentage
µ	-micro

CHAPTER 1

INTRODUCTION

1.0 Research background and outline

Nanoparticles are an important aspect in the field of nanobiotechnology, a cross-over between nanotechnology- and biology-related fields to create materials for studying biological systems (Gazit, 2007). It can be defined as particles within the size range of 1 and 100 nm and shows unique properties that differ from those observed in bulk samples of the same material (Auffan *et al*., 2009). In the recent years, chitosan nanoparticles have received considerable attention due to its low toxicity as well as its biodegradable and biocompatible properties, which forms an ideal hydrophilic carrier system (Hirano, 1996). Chitosan is usually derived from a naturally occurring substance known as chitin usually found in the exoskeletons of insects, crustaceans and some cell walls of certain fungi species (Jia *et al.*, 2009). Methods that can be used to prepare chitosan nanoparticles include ionic cross-linking, polymerization, precipitation and self-assembly (Wang *et al.*, 2011). The co-administration of chitosan and its derivatives have led to an improved bioavailability of various pre-orally given drugs such as doxorubicin hydrochloride and insulin (Takeuchi *et al.*, 2003).

The use of chitosan nanoparticles as efficient drug and gene delivery systems requires that its uptake effectiveness into its target site to be assessed to confirm successful cellular internalization. In addition, its accumulation efficiency inside cells at different time points is also another important aspect that needs to be studied to ensure efficient drug release or retention inside cells. This can be ascertained through the *in vitro* labeling of chitosan nanoparticles with suitable markers such as FITC to visualize and track cellular localization (Jia *et al.*, 2009). Also, the efficiency of accumulation inside cells after cellular uptake at different time points has to be taken into account in order to ensure efficient drug release or retention as desired. FITC is a type of fluorophore derived from fluorescein which that emits green fluorescence and is usually covalently attached onto polysaccharide molecules such as chitosan, alginate, dextran and starch (Chin *et al.*, 2014). In previous years,

fluorophores were physically entrapped for incorporation into nanoparticles due to its simplicity but this has caused major drawbacks such as fluorophore leaching leading to cell toxicity, contamination of samples and incorrect signal measurements. Therefore, the recent approach of covalent attachment was proven to reduce fluorophore leaching, improved photostability, more stable fluorescent signals and enhanced lifetime FITC as compared to physical entrapment of fluorophores (Schulz *et al*., 2009).

In this study, chitosan nanoparticles were synthesized using ionic cross-linking route and then labeled with the FITC dye for *in vitro* visualization and localization into 786-O human kidney cancer cell line using fluorescence microscopy techniques. The chitosan nanoparticles were characterized using dynamic light scattering (DLS) for particle size distribution and polydispersity index evaluation. Finally, the nanoparticles were labeled with FITC, followed by treatment of human kidney cancer cells *in vitro* and visualization using fluorescence microscopy.

1.2 OBJECTIVES

There are basically three objectives of this project, as mentioned below:

1. To synthesize chitosan nanoparticles using ionic gelation method

2. To label the chitosan nanoparticles with FITC dye for visualization and localization inside human kidney cancer cells

3. To observe the uptake of FITC-labeled chitosan nanoparticles into human cancer kidney cells.

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