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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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HEPATITIS B CAPSID AS A DELIVERY VEHICLE INTO MAMMALIAN CELLS

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August 2012

Chair: **Professor Tan Wen Siang, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

The recombinant hepatitis B virus (HBV) core antigen (HBcAg) expressed in *Escherichia coli* self-assembles into icosahedral virus-like particles (VLPs) of about 35 nm. It possesses a range of surface chemistries for recombinant DNA alteration and chemical modification to display cell-targeting signals on its surface. The HBcAg nanoparticles could serve as a potential nano-container to package therapeutic molecules for drug and gene delivery. In this study, the association, dissociation and the molecule packaging properties of the C-terminally truncated HBcAg (tHBcAg) were studied with protein denaturants. Light scattering analysis and transmission electron microscopy (TEM) revealed that the dissociated tHBcAg was able to re-associate into nanoparticles when the denaturants were removed. In order to evaluate the potential of the nanoparticles in capturing molecules, the green fluorescent protein (GFP) was used as a model for encapsidation. Data showed that

the GFP molecules were packaged and remained active in the tHBcAg nanoparticles after the dissociation and re-association steps.

The insertion of foreign peptides at the N-terminal end of HBcAg displays the inserts on the surface of the nanoparticles. In order to develop the tHBcAg nanoparticles as a cell specific delivery system, the HBV $preS1_{1-108}$ region was employed as a ligand to target the human hepatocytes. The $preS1_{1-108}$ region was fused to the N-terminal end of the His_6HBcAg (preS1His_6HBcAg) via recombinant DNA technique. However, the fusion protein was insoluble in *E. coli* and did not form VLPs. A method to reconstruct and to display the preS1 on the tHBcAg nanoparticle was established by dissociating an appropriate molar ratio of the tHBcAg to the preS1His_6HBcAg in urea. Gold immuno-TEM showed that the subunit mixture re-assembled into icosahedral nanoparticles, displaying the preS1 ligand on the surface of VLPs. In order to assess the application of the reconstructed VLPs (rVLPs) as a delivery vehicle, fluorescein molecules were cross-linked on the tHBcAg of the rVLPs and tested on hepatocytes *in vitro*. Fluorescence microscopy revealed that the fluorescent rVLPs were internalised into HepG2 cells, efficiently.

The peptide, SLLGRMKGA has been shown to bind and chemically cross-link the aspartic acid residues (D_{64} and D_{78}) or glutamic acid residue (E_{77}) at the tips of tHBcAg nanoparticles. The following study reports an extension of this idea as a universal 'nano-glue' to display different cell-internalising peptide (CIP) at the tips of tHBcAg nanoparticles. As a model of our studies, peptide ligand that internalised HeLa cells were selected from a random 12-mers peptide library displayed on

filamentous M13 phage. Immuno-fluorescence microscopy showed that the isolated M13 clones, displaying HTLQIPQHATSF, KLHISKDHIYPT and THASKNTSYFTV internalised HeLa cells specifically. The isolated peptide (KLHISKDHIYPT) was co-synthesised with the 'nano-glue' sequence (KLHISKDHIYPT**GGG**SLLGRMKGA; JG24) and conjugated chemically to the tHBcAg nanoparticles using cross-linkers (EDC and sulfo-NHS). Transmission electron microscopy (TEM) revealed that the tHBcAg nanoparticles remained intact after the conjugation. Interestingly, when the peptide was conjugated to the fluorescein labelled tHBcAg (FtHBcAg) nanoparticles, the nanoparticles internalised HeLa cells and the fluorescent was observed under a fluorescence microscope. tHBcAg nanoparticles were later used to packaged fluorescent oligonucleotide, conjugated with cell-targeting peptide and added to HeLa cells. Fluorescent microscopy revealed the delivery of the oligonucleotide into the cells.

The tHBcAg nanoparticles could serve as a potential nano-vehicle to package molecules and to target various cells specifically by displaying different cell specific ligands at the N-terminal region or having the CIPs conjugated chemically at the tips of tHBcAg nanoparticles.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KAPSID HEPATITIS B SEBAGAI KENDERAAN PENGHANTARAN KE DALAM SEL-SEL MAMALIA

Oleh

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Ogos 2012

Pengerusi: **Profesor Tan Wen Siang, PhD**

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Antigen teras (HBcAg) rekombinan virus hepatitis B (HBV) yang diekspres dalam *Escherichia coli* dapat berinteraksi sesama sendiri menjadi kapsid icosahedral dengan ukuran lebih kurang 35 nm. Kapsid tersebut mempunyai permukaan untuk modifikasi DNA rekombinan dan pengubahsuaian secara kimia demi mempamerkan isyarat-isyarat sasaran sel pada permukaannya. Partikel-partikel nano HBcAg tersebut sesuai digunakan sebagai bekas-nano yang berpotensi dalam pembungkusan molekul-molekul terapeutik untuk kegunaan penghantaran gen dan ubat. Dalam kajian ini, sifat-sifat persatuan, penceraian dan pembungkusan molekul HBcAg yang dipendekkan di terminal-C (tHBcAg) telah dikaji dengan penyahasli-penyahasli protein. Analisis penaburan cahaya dan mikroskopi penyiaran elektron (TEM) mendedahkan bahawa tHBcAg yang tercerai tersebut dapat bersatu semula kepada partikel-partikel nano aslinya selepas penyahasli-penyahasli disingkirkan. Demi menilai potensi partikel-partikel nano tersebut dalam pembungkusan molekul-

molekul, protein berpendarfluor hijau (GFP) telah digunakan sebagai model dalam pembungkusan. Data menunjukkan bahawa molekul-molekul GFP terbungkus dan kekal aktif di dalam partikel-partikel nano tHBcAg selepas langkah-langkah perceraian dan penghimpunan semula.

Penyisipan peptida-peptida asing di bahagian terminal-N HBcAg mempamerkan sisipan-sisipan tersebut pada permukaan partikel-partikel menyerupai virus (VLPs). Dalam kajian ini, bahagian $preS1_{1-108}$ telah digunakan sebagai ligan sasaran sel-sel hati manusia. Bahagian $preS1_{1-108}$ tersebut telah dicantumkan pada hujung terminal-N His_6HBcAg ($preS1His_6$HBcAg) melalui teknik rekombinan DNA. Akan tetapi, hasil protein rekombinan tersebut tidak larut di dalam *E. coli* dan tidak membentuk VLPs. Satu kaedah untuk pembinaan semula dan pempameran preS1 pada partikel-partikel nano telah ditubuhkan dengan penceraian nisbah molar tHBcAg kepada $preS1His_6$HBcAg yang bersesuaian dalam larutan urea. TEM-imuno emas menunjukkan bahawa campuran subunit-subunit tersebut dapat berhimpun semula menjadi partikel-partikel nano icosahedral serta mempamerkan ligan preS1 tersebut di permukaan VLPs. Dalam kajian aplikasi VLPs yang terbina semula (rVLPs) tersebut untuk kegunaan sebagai kenderaan penghantaran, tHBcAg di dalam rVLPs tersebut telah dilabelkan dengan molekul-molekul "fluorescein" dan diuji dengan sel-sel hati manusia secara *in vitro*. Mikroskopi berpendarfluoran menunjukkan bahawa rVLPs berpendarfluor tersebut dapat menembusi sel-sel HepG2 dengan berkesan.

Sebelum ini, kajian telah menunjukkan bahawa peptida SLLGRMKGA dapat mengikat dan disambung-silang secara kimia pada residu-residu asid aspartik (D_{64}

and D_{78}) atau residu asid glutamic (E_{77}) di hujung-hujung partikel nano tHBcAg. Di sini, kami melaporkan satu unjuran tersebut sebagai 'glu-nano' umum untuk mempamerkan pelbagai peptida penembusi sel (CIP) pada hujung-hujung partikel nano tHBcAg. Sebagai satu model dalam kajian tersebut, ligan peptida yang menembusi sel-sel HeLa telah dipilih dari satu perpustakaan peptida rawak 12-mer yang terpamer pada faj M13. Mikroskopi-imuno berpendarfluoran menunjukkan bahawa klon-klon M13 terpencil yang memaparkan peptida-peptida HTLQIPQHATSF, KLHISKDHIYPT dan THASKNTSYFTV yang sewajarnya dapat menembusi secara khususnya ke dalam sel-sel HeLa. KLHISKDHIYPT disintesis bersama dengan urutan 'glu-nano' (KLHISKDHIYPT**GGG**SLLGRMKGA; JG24) dan dikonjugasikan secara kimia pada partikel-partikel nano tHBcAg dengan menggunakan penyambung-silang (EDC dan sulfo-NHS). Permerhatian TEM mendedahkan bahawa partikel-partikel tHBcAg tersebut dapat mengekalkan kesempurnaannya selepas proses konjugasi. Apabila peptida JG24 dikonjugasikan pada partikel-partikel nano tHBcAg yang dilabel dengan fluorescein, partikel-partikel nano tersebut menembus ke dalam sel-sel HeLa dan pendarfluoran sel-sel tersebut dapat diperhatikan di bawah mikroscopi berpendarfluoran. Selepas itu, partikel-partikel nano tHBcAg digunakan untuk membungkus oligonukleotida berpendarfluor, konjugasi dengan peptide-peptida sasaran sel, dan ditambahkan dalam sel-sel HeLa. Mikroscopi berpendarfluoran menunjukkan penghantaran oligonukleotida-oligonukleotida ke dalam sel-sel tersebut.

Partikel-partikel nano tHBcAg dapat digunakan sebagai kenderaan-nano yang berpotensi untuk membungkus molekul-molekul dan mensasar ke pelbagai sel yang

tertentu dengan pempameran ligan-ligan sel yang khusus sama ada di bahagian terminal-N ataupun secara konjugasi kimia di puncak-puncak partikel nano tHBcAg.

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I certify that an Examination Committee has met on **August 2012** to conduct the final examination of **Lee Khai Wooi** on his degree thesis entitled "**Hepatitis B capsid as a delivery vehicle into mammalian cells**" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the student be awarded the Degree of Doctor of Philosophy.

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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 **Doctor of Philosophy**. 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, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

LEE KHAI WOOI

Date:

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