



UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF BIOSENSOR PROBE FOR THE DETECTION OF MALACHITE GREEN AND LEUCO-MALACHITE GREEN FOR APPLICATION IN FISHERY INDUSTRY

NURUL HIDAYAH BINTI AHMAD PUAT

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**MASTER OF SCIENCE
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UNIVERSITI PUTRA MALAYSIA
BERILMU BERBAKTI

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By

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**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfillment of the Requirements for the Degree of Master of Science**

December 2013

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

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December 2013

Chairperson: Professor Fatimah Abu Bakar, PhD
Faculty: Science and Food Technology

The use of Malachite Green (MG) as an anti-fungal and anti-bacterial in the aquaculture industry has obtained attention in food safety. MG and its metabolite Leuco-Malachite Green (LMG) are highly toxic to aquatic environment and harmful to human health through daily consumption and it becomes more dangerous when accumulated in fish tissues. At present, the minimum required performance limits (MRPLs) for total MG (MG and LMG) concentration is $2 \mu\text{gkg}^{-1}$ or 2 ppb. Hence, the simple, rapid, sensitive and portable biosensor is really needed.

The aim of this research is to study the chemical inhibition of Butyrylcholinesterase enzyme (BuChE) by total MG in the presence of 0.3 mM Butyrylthiocholine iodide substrate (BTCi) for MG biosensor development. The MG biosensor has developed for total MG detection in fishes especially the tilapia and has validated by using the LC-MS/MS method. This electrochemical study has done by using screen-printed carbon electrode (SPCE) and the inhibition study has done by using free and immobilized enzyme. Then, it has characterized and analyzed using cyclic voltammetry (CV) and chrono-amperometry (CM). The supporting electrolyte, pH, set potential, scan rate and response range includes enzyme loading, polymer concentration, incubation and response time of the MG biosensor has optimized electrochemically. Meanwhile, the reproducibility, repeatability, operational stability and storage stability included cross reactivity has carried out. Finally, the developed MG biosensor method was validated with the LC-MS/MS method using real fish samples including the recovery study.

In this study, 4U mL^{-1} BuChE enzyme (C1057) has used and the CV analysis was carried out as a preliminary study. The reproducibility of the SPCE was characterized electrochemically against potassium hexacyanoferrate (II) trihydrate and 93.65 % active surface areas of carbon working electrode were achieved. BuChE enzyme has incorporated within 0.08M pyrrole monomer during the electro-polymerization process at 0.1 V amperometrically for 20 minutes, which enzyme has entrapped within the thin films of polypyrrole (PPy). The total MG (MG and LMG) has determined by measuring

the current using amperometric technique at 0.4 V for 100 s using 0.1 M phosphate buffer at pH 8.0. This analysis needs five minutes of incubation time for enzyme-substrate reaction and inhibition before measurement, and it may get up to 78 % inhibition at 2 ppb total MG.

A linear standard curve of total MG has developed (0.25 ppb to 10 ppb) based on the current measurement (μA) using standard solution ($Y = -0.9113x + 10.84$, $R^2 = 0.9445$), which has good reproducibility and operational stability until five measurements. Instead of that, the enzyme activity has reduced (repeatability) slowly after the third measurement. However, the MG biosensor probe is able to re-use after treated with the pyridine-2-aldoimine (PAM-2) activator. The shelf life of the MG biosensor took more than six months with 20 % protein or enzyme loss. The total MG also showed the higher inhibition (48 %) at 2 ppb compared to other triphenylmethane dyes. This MG biosensor method has validated using the LC-MS/MS method with a regression value of 0.9262 (correlation graph) upon ten unknown samples with recoveries valuing more than 60 % of spiked sample.

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

**PEMBANGUNAN BIOPENDERIA PENGESAN UNTUK TUJUAN
PENGESANAN MALAKIT HIJAU DAN LEUKO-MALAKIT HIJAU UNTUK
APLIKASI DALAM INDUSTRI PERIKANAN**

Oleh

NURUL HIDAYAH BINTI AHMAD PUAT
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Penggunaan 'Malachite Green' (MG) sebagai anti-kulat dan anti-bakteria di dalam industri perikanan telah mendapat perhatian dalam keselamatan makanan. MG dan metabolit 'Leuco-Malachite Green' (LMG) adalah sangat toksik kepada persekitaran akuatik dan berbahaya kepada kesihatan manusia melalui penggunaan harian dan ia menjadi lebih berbahaya apabila terkumpul di dalam tisu ikan. Pada masa ini, had prestasi minimum yang diperlukan (MRPLs) bagi kepekatan keseluruhan MG (MG dan LMG) adalah $2 \mu\text{gkg}^{-1}$ atau 2 ppb. Oleh itu, biopenderia yang mudah, cepat, sensitif dan mudah alih adalah benar-benar diperlukan.

Matlamat penyelidikan ini adalah untuk mengkaji perencatan kimia enzim 'Butyrylcholinesterase' (BuChE) oleh keseluruhan MG dengan kehadiran 0.3 mM 'Butyrylthiocholine' substrat (BTCi) untuk pembangunan biopenderia MG. Biopenderia MG ini dibangunkan untuk pengesanan keseluruhan MG dalam ikan terutama ikan tilapia dan ia akan disahkan dengan menggunakan kaedah kromatografi cecair-seiring spektrometri jisim (LC-MS/MS). Kajian elektrokimia ini telah dijalankan menggunakan elektrod bercetak skrin karbon (SPCE) dan kajian perencatan telah dijalankan menggunakan enzim bebas dan pegun. Ia dicirikan dan dianalisis masing-masing dengan menggunakan kaedah kitar voltametri (CV) dan chrono-amperometri (CM). Elektrolit sokongan, pH, set keupayaan, kadar imbasan dan julat tindak balas termasuk muatan enzim, kepekatan polimer, masa inkubasi dan tindak balas biopenderia MG telah dioptimumkan secara elektrokimia. Kemudian, ia telah dicirikan dalam segi kebolehasilan, kebolehulangan, kestabilan operasi dan penyimpanan termasuk kereaktifan silang. Akhirnya, kaedah biopenderia MG yang telah berjaya dibangunkan ini disahkan dengan kaedah LC-MS/MS menggunakan sampel ikan sebenar termasuk kajian mendapatkan semula.

Dalam kajian ini, 4 U mL^{-1} enzim BuChE (C1057) telah digunakan dan analisis CV telah dijalankan sebagai satu kajian awal. Kebolehasilan SPCE telah dicirikan secara

elektrokimia terhadap 'potassium hexacyanoferrate (II) trihydrate' dan 93.65 % kawasan aktif permukaan elektrod karbon kerja telah dicapai. BuChE telah digabungkan dalam 0.08 M 'pyrrole monomer' semasa proses elektro-pemolimeran pada 0.1 V secara amperometri selama 20 minit, yang mana enzim telah terperangkap dalam lapisan nipis filem 'polypyrrole' (PPy). Keseluruhan MG telah ditentukan dengan mengukur arus menggunakan teknik amperometri pada 0.4 V selama 100 saat menggunakan 0.1 M penimbal fosfat pada pH 8.0. Analisis ini memerlukan lima minit masa pengeraman bagi tindak balas enzim-substrat dan perencatan sebelum pengukuran, dan ia boleh mencapai sehingga 78 % perencatan pada kepekatan 2 ppb keseluruhan MG.

Satu lengkung linear piawai keseluruhan MG telah dibangunkan (0.25 ppb hingga 10 ppb) berdasarkan pengukuran arus elektrik (μA) menggunakan larutan piawai ($Y = -0.9113x + 10.84$, $R^2 = 0.9445$), yang mana mempunyai kebolehulangan yang baik dan kestabilan operasi sehingga lima kali pengukuran. Sebaliknya, aktiviti enzim akan berkurang (kebolehulangan) secara perlahan-lahan selepas bacaan ketiga. Walaubagaimanapun, siasatan biopenderia MG boleh digunakan semula selepas dirawat dengan pengaktif 'pyridine-2-aldoamine' (PAM-2). Jangka hayat biopenderia MG adalah lebih daripada enam bulan dengan kehilangan 20 % protein atau enzim. Keseluruhan MG juga menunjukkan perencatan yang tinggi (48 %) pada kepekatan 2 ppb berbanding pewarna 'triphenylmethane' lain. Kaedah biopenderia MG telah disahkan dengan menggunakan kaedah LC-MS/MS dengan nilai regrasi 0.9262 (graf korelasi) terhadap sepuluh sampel yang tidak diketahui dengan nilai dapatan semula lebih daripada 60 % 'spiked' sampel.

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I certify that a Thesis Examination Committee has met on, (17 December 2013) to conduct the final examination of Nurul Hidayah Binti Ahmad Puat on her thesis entitled “Development of Biosensor Probe for the Detection of Malachite Green and Leuco-Malachite Green for Application in Fishery Industry” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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DECLARATION

Declaration by graduate student

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