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DEVELOPMENT OF A URIC ACID BIOSENSOR USING URICASE-IMMOBILIZED GRAPHENE OXIDE

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By

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February 2017

Chairman : Asilah Ahmad Tajudin, PhD
Faculty : Biotechnology and Biomolecular Sciences

High level of uric acid in the body will cause various diseases for instance gout, Lesch-Nyhan syndrome, cardiovascular and neurological diseases. Currently, the diagnostic applications of uric acid detection are time consuming, lab-based and not practical in terms of continuous monitoring. Therefore, improvements in the accuracy, detection time and sensitivity of these measurements can be done through the use of biosensors. The aim of this study was to develop a fast, high sensitivity and specificity uric acid biosensor through the use of uricase-immobilized graphene oxide. Uricase or urate oxidase was used as a catalyst in the oxidation of uric acid into allantoin and form by-products of hydrogen peroxide and carbon dioxide. The uricase was immobilized onto a carbon-composed platform of graphene oxide (GO). GO is a two-dimensional (2D) single layer of carbon with many active functional groups. GO was used in this study because of its unique properties (large surface area, good biocompatibilityand mechanical flexibility). The GO was synthesized by using a simplified Hummers' method. Then it was characterized using ultraviolet-visible spectroscopy (UV-Vis), X-ray diffraction (XRD), and field emission electron microscopy (FESEM) and showed a GO with typical characteristics similar to GO formed via other methods of synthesis. Next, uricase was immobilized onto the GO to test the enzyme functionality as a bioreceptor for uric acid detection. EDC-NHS ester was used as a crosslinking reagent to chemically modify the GO. The immobilized uricase showed enzyme activity that was comparable to the free enzyme with 88% activity retained. Again, the modified GO-uricase (GOU) was characterized using FESEM, XRD and energy-dispersive x-ray spectroscopy (EDX). Through FESEM, both modified GO with EDC-NHS and modified GO with immobilized uricase showed a typical FESEM image of GO as reported in the literature. XRD indicated that the uricase may have blocked the peaks

of GO and ITO glass due to its large structure. From the EDX data, carbon and oxygen compositions are abundant in the GOU compound along with other molecules for example nitrogen and sodium. Then, the electrocatalytic detection of uric acid (UA) was carried out for the GOU via cyclic voltammetry (CV) using a potentiostat. Hence, the GOU was adhered to a glassy carbon electrode (GCE) to facilitate the redox reaction between the enzyme and the substrate. The electrocatalytic response exhibited a linear dependence on the UA concentration ranging from 0.02 mM to 0.49 mM with a detection limit of 3.45 μM at the signal-to-noise ratio of 3 for CV and 6.37 μM at the signal-to-noise ratio of 3 for chronoamperometry (CA). A selectivity study using ascorbic acid (AA) also was carried out to determine the specificity of the sensor in detecting uric acid even in the presence of other interfering compound. Through the CV, AA did not interfere in the UA detection as it formed its own oxidation peak at 0.15 V whilst oxidation peak for UA at 0.47 V and the oxidation peak of UA is much higher as compared to AA. This indicated that the biosensor was also highly selective towards UA. The biosensor also exhibited a good stability when subjected to stability test using different scan rates. It was able to retain its CV pattern with a distinctive peak of oxidation of UA. Lastly, reproducibility test was carried for the GOU and only 15% reduction of peak current of UA upon observation after 10 days was found. In conclusion, the developed biosensor showed promising results as it was able to detect UA both in uric acid-spiked samples and in the presence of other interfering compound.

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

PEMBANGUNAN BIOSENSOR ASID URIK MENGGUNAKAN IMOBILISASI URIKASE-GRAFIN OKSIDA

Oleh

MUHAMAD NADZMI BIN OMAR

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Pengerusi : Asilah Ahmad Tajudin, PhD
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Tahap tinggi asid urik di dalam badan akan menyebabkan beberapa penyakit seperti gout, sindrom *Lesch-Nyhan*, penyakit kardiovaskular dan neurologi. Pada masa ini, aplikasi diagnostik untuk pengesanan asid urik adalah memakan masa, dijalankan di makmal dan tidak praktikal dari segi pemantauan berterusan. Oleh itu, penambahbaikan dalam ketepatan, masa pengesanan dan kepekaan pengukuran ini boleh dilakukan melalui penggunaan biosensor. Tujuan kajian ini adalah untuk membangunkan biosensor asid urik yang pantas, tinggi sensitiviti dan spesifikasi mengunakan imobilisasi urikase-grafin oksida. Urikase atau uriko-oksidase telah digunakan sebagai pemangkin dalam pengoksidaan asid urik ke alantoin dan bentuk produk sampingan hidrogen peroksida dan karbon dioksida. Urikase telah diimobilisasi ke atas sebuah platform yang diperbuat daripada karbon dipanggil grafin oksida (GO). GO adalah satu atom tebal dengan 2 dimensi (2D) karbon atom berlapisan tunggal yang mengandungi pelbagai kumpulan berfungsi aktif. GO telah digunakan dalam kajian ini kerana ciri-cirinya yang unik (kawasan permukaan yang besar, bioserasi dan fleksibiliti mekanikal yang baik). GO telah disintesis menggunakan kaedah Hummers yang dipermudahkan. GO ini kemudiannya dicirikan menggunakan spektroskopi ultraviolet-benderang (UV-Vis), mikroskop elektron pengimbas pancaran medan (FESEM) serta pembelauan sinar-X (XRD) dan menunjukkan ciri-ciri GO yang sama seperti kaedah sintesis lain. Seterusnya, enzim urikase telah diimobilisasi keatas GO untuk menguji fungsi enzim sebagai bioreseptor bagi pengesanan asid urik. Ester EDC-NHS digunakan sebagai reagen silang untuk mengubah suai GO secara kimia. Urikase yang telah diimobilisasi menunjukkan aktiviti enzim yang setanding dengan enzim bebas, dengan aktiviti 88% dikekalkan. Sekali lagi, GO-urikase (GOU) yang diubahsuai telah dicirikan menggunakan FESEM, XRD dan spektroskopitenaga-serakan

x-ray (EDX). Melalui FESEM, kedua-dua GO yang diubahsuai dengan EDC-NHS dan urikase menunjukkan imej tipikal FESEM GO seperti yang telah dilaporkan dalam literatur. XRD telah melaporkan bahawa enzim urikase telah menghalang puncak GO dan ITO glass disebabkan strukturnya yang besar. Daripada data EDX, komposisi karbon dan oksigen banyak terdapat dalam GOU bersama-sama dengan molekul lain seperti nitrogen dan sodium. Kemudian, urikase diimobilisasi pada GO tertakluk kepada pengesanan elektrokatalitik asid urik (UA) melalui voltammetri berkitar (CV) menggunakan potentiostat. Atas sebab itu, elektrod karbon berkaca (GCE) telah diubahsuai dengan meletakkan GO bersama-sama dengan urikase yang diimobilisasi untuk memudahkan tindak balas redoks antara enzim dan substrat. Respon eletrokatalitik menunjukkan pergantungan linear kepada kepekatan UA yang terdiri daripada 0.02 mM kepada 0.49 mM dengan had pengesanan 3.45 μM pada 3 isyarat kepada nisbah bunyi bagi CV dan 6.37 μM pada 3 isyarat kepada nisbah bunyi bagi kronoamperometri (CA). Satu kajian selektif menggunakan asid askorbik (AA) juga telah dijalankan untuk menentukan spesifikasi sensor dalam mengesan asid urik walaupun terdapat kompaun mengganggu lain. Melalui CV, AA didapati tidak mengganggu dalam pengesanan UA kerana ia membentuk puncak pengoksidaan sendiri pada 0.15 V manakala puncak pengoksidaan untuk UA pada 0.47 V dan puncak pengoksidaan bagi UA lebih tertinggi berbanding AA. Ini menunjukkan biosensor tersbut lebih selektif terhadap UA. Biosensor ini juga mempamerkan stabiliti yang baik apabila tertakluk kepada kajian stabiliti menggunakan beberapa kadar pengesanan yang berbeza. Ia dapat mengekalkan corak CV dengan puncak pengoksidaan UA tersendiri. Akhir sekali, ujian kebolehulangan telah dijalankan untuk GOU dan hanya pengurangan 15% kemuncak UA atas pemerhatian selepas 10 hari. Kesimpulannya, biosensor yang dibangunkan ini menunjukkan hasil yang memberangsangkan kerana ia dapat mengesan UA di kedua-dua sampel asid urik yang dilonjak kedalam dan dengan kehadiran komponen gangguan lain.

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NADZMI OMAR

I certify that a Thesis Examination Committee has met on (Date of viva voce) to conduct the final examination of Muhamad Nadzmi bin Omar on his thesis entitled "Development of a Uric Acid Biosensor using Uricase-Immobilized Graphene Oxide" 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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This is to confirm that:

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%	Percent
°C	Degree Celsius
2θ	2 theta/180^{o}
A	Ampere
d	Diameter
et al.	And others
g	Gram
h	Hours
kDa	Kilo Dalton
M	Molar
mg	Milligram
min	Minutes
ml	Milliliter
mM	Millimolar
mV	Millivolt
mV/s	Millivolt per second
nM	Nanomolar
nm	Nanometer
nmol/min	Nanomol per minute
^{o}	Degree
Ø	Diameter
pH	Exponential of the concentration of hydrogen ion
pmol/min	Picomol per minute
rpm	Revolutions per minute
s	Seconds

U	Unit
U/mg	Unit per milligram
U/ml	Unit per millilitre
V	Voltage
w/v	Weight per volume
µA	Microampere
µg	Microgram
µl	Microliter
µM	Micromolar

CHAPTER 1

INTRODUCTION

The diagnosis of some diseases can be done by determining and monitoring the concentration of uric acid (UA) in bodily fluids such as serum and urine, and because of that makes it a valuable diagnostic indicator.The primary end product of the purine metabolism in the body is UA, with the usual physiological levels in serum to be between 0.13 mM and 0.46 mM (2.18-77 mg dl^{-1}) (Raj and Ohsaka, 2003; Huang et al., 2004). The surplus in the production of UA in serum has been linked to both cardiovascular and neurological diseases while an imbalance of UA levels in the body can cause other diseases such as gout, hyperuricemia and Lesh-Nyhan syndrome (Moallem et al., 2002; Baker et al., 2005). By coupling uricase with 4-aminoantipyrine-peroxidase it becomes one of the most useful enzymatic methods in determining the levels of UA (Gochman and Schmitz, 1971).Uricase acts as a catalyst in the oxidation of UA where it oxidizes the purine ring into allantoin, carbon dioxide, and hydrogen peroxide (Motojima et al., 1988). Uricase is present in a majority of organisms where it acts in various metabolic roles depending on its host organism, but is not present in the human body due to gene evolution (Wu et al., 1989).

Most UA detection are electrochemical (Hoshi et al., 2003; Chen et al., 2005; El Bouhouti et al., 2009), UV and colorimetric (Tetsuo and Yukiko, 1976; Schrenkhammer and Wolfbeis, 2008), chemiluminescence (Yao et al., 2003; Yang and Zhang, 2010; Yu et al., 2011), and optical (Grabowska et al., 2008). However, these methods provide limited applications due to their drawbacks (sensitivity, cost, interferences, and selectivity). Because of this, methodologies in electrochemical biosensing are now based upon various nanostructures such as carbon nanotubes, graphene, and nonporous materials. These materials possess exceptional properties such as their high electrical conductivity, high sensitivity, low cost, and non-toxicity, which have garnered a large interest in them (Ram et al, 2000; Dixon et al., 2002; Jianrong et al., 2004). Graphene oxide (GO) possesses many incredible properties (large specific surface area, high dispersibility in water, and an abundance of oxygen functional groups) which provides many promising applications (Li et al., 2008; Park and Ruoff, 2009; Liu et al., 2010; Veerapandian et al., 2012). One of its potential applications is that it can act as a biosensing medium. This is due to its low toxicity in bodily conditions, inertia, and great biocompatibility (Wang et al., 2011; Wu et al., 2012).

Combination of immobilized enzyme with electrochemical biosensing provides unique advantages as they can maintain the enzymatic activity in a microenvironment and enhance the direct electron transfer between the active sites and the electrode (Ali et al., 2011). However, as the method of enzyme immobilization can affect not only the sensitivity and specificity of the analysis, the structure of the immobilized enzyme and also the shelf-life, it is imperative that a researcher needs to find one that is suitable for the intended application. Numerous methods of immobilization of uricase onto various supports have been reported such as adsorption onto cellulose acetate membrane (Kan et al., 2004), polyaniline film (Yao et al., 2003), physisorption onto polypyrrole and polyaniline (Arslan, 2008), crosslinking onto chitosan membrane (Martinez-Pérez et al., 2003) and zinc oxide nanorods (Zhang et al., 2004). However these methods were complicated and provide less stability that limit the fabrication and application of uric acid biosensors.

In this study, we have reported the synthesis of GO through simplified Hummer's method and a simple immobilization approach of uricase onto GO (GOU) using EDC-NHS esters as a potentiometric uric acid biosensor. The fabricated GOU electrode was also characterized by XRD and FESEM. Basic principle of uricase assay was conducted to test the immobilized enzyme activity and functionality. The electrocatalytic analysis of the GOU sensor on the detection of UA was investigated through cyclic voltammetry. This development shows promising features for examples; low cost, simple design, highly sensitive and specific, fast response, less interference and ease of operation of a uric acid biosensor.

The objectives of the study were:

1. To immobilize uricase onto graphene oxide surface.
2. To characterize the uricase immobilized-graphene oxide using FESEM, XRD and EDX.
3. To test the sensor on uric acid detection at clinically significant levels from uric acid-spiked samples.

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