DEVELOPMENT OF ANTI-HEPATITIS B VIRUS CORE ANTIGEN BIOSENSOR USING GOLD NANOPARTICLE-DECORATED REDUCED GRAPHENE OXIDE

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By

MOHAMAD FARID BIN ABD MUAIN

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February 2019

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

The presence of antibody against hepatitis B virus core antigen (anti-HBcAg) in blood serum indicates that the patients have been exposed to hepatitis B virus (HBV), and can be used as a biomarker for occult hepatitis B virus infection (OBI). Currently, the diagnostic applications targeting HBV infection are time-consuming, lab-based and not practical for fast point-of-care (POC) diagnostic. Therefore, future development of HBV infection detection systems for rapid, and highly sensitive method is imperative. This work focused on the development of an immunosensor for the detection of anti-HBcAg through the use of hepatitis B virus core antigen (HBcAg) as the main bioreceptor. This bioreceptor was immobilized onto the gold nanoparticles-decorated reduced graphene oxide (rGO-en-AuNPs) which possesses excellent electrical conductivity and ability to hold highest amount of HBcAg compared to graphene oxide (GO) and reduced graphene oxide (rGO-en). Fabrication of rGO-en-AuNPs employed ethylenediamine as the reducing agent of GO, and also act as agent to hold the gold nanoparticles (AuNPs) in place on the rGO. All four types of characterization which are field emission scanning electron microscope (FESEM), X-ray diffraction (XRD), Fourier transform infrared (FTIR) and Raman spectroscopy confirmed that GO had been reduced and AuNPs had successfully incorporated onto the rGO-en. Then, the assembled HBcAg-immobilized rGO-en-AuNPs on the screen-printed electrode (SPE) was allowed to undergo impedimetric detection of anti-HBcAg with the concentration ranging from 3.91 ng mL^{-1} to 125.00 ng mL^{-1}, with antibody against estradiol (anti-estradiol) and bovine serum albumin (BSA) as the interferences. Upon successful detection of anti-HBcAg in spiked buffer samples with the limit of detection (LOD) of 3.80 ng mL^{-1} at 3 σ m^{-1} (linear regression equation of ΔR_{ct} = 0.0261[anti-HBcAg] + 6.421 (R^2 = 0.982)), impedimetric detection of the antibody was then further carried out in spiked human serum samples. In the presence of interferences, and the human serum samples, the LOD were calculated to be at 3.88 ng mL^{-1} at 3 σ m^{-1} (linear regression equation of ΔR_{ct} = 0.0315[anti-HBcAg] + 6.309 (R^2 = 0.971)), and 5.60

ng mL^{-1} at 3 σ m^{-1} (linear regression equation of ΔR_{ct} = 0.0345[anti-HBcAg] + 6.467 (R^2 = 0.961)) respectively. The electrochemical response in all anti-HBcAg detection showed a linear relationship between electron transfer resistance (R_{ct}) and the concentration of anti-HBcAg. In contrast, no cross reaction observed when the HBcAg-immobilized rGO-en-AuNPs was allowed to react with non-related antibody. This stage of biosensor development is a primary platform for further study in producing the anti-HBcAg immunosensor prototype that possesses higher and better selectivity and specificity in the future. Further miniaturization works are possible to be carried out given the chosen method of electrochemistry.

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

PEMBANGUNAN BIOSENSOR ANTIBODI BAGI ANTIGEN TERAS VIRUS HEPATITIS B MENGGUNAKAN GRAFIN OKSIDA TERTURUN YANG DIHIASI DENGAN NANOPARTIKEL EMAS

Oleh

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Kehadiran antibodi yang menentang antigen teras virus hepatitis B (anti-HBcAg) di dalam serum darah menandakan bahawa pesakit telah terdedah kepada virus hepatitis B (HBV), dan boleh menjadi penanda biologi untuk jangkitan virus hepatitis B tertutup (OBI). Kini, aplikasi-aplikasi diagnostik HBV memakan masa, berasaskan makmal dan tidak praktikal untuk diagnostik semasa penjagaan (POC) yang cepat. Oleh itu, pembangunan masa hadapan untuk sistem pengesanan jangkitan HBV yang cepat, mempunyai kesensitifan yang tinggi adalah penting. Kajian ini memfokuskan kepada pembangunan penderia imun bagi mengesan anti-HBcAg dengan menggunakan antigen teras virus hepatitis B (HBcAg) sebagai bioreseptor utama. Bioreseptor ini telah dinyahgerakkan ke atas grafin oksida terturun yang dihiasi dengan nanopartikel emas (rGO-en-AuNPs) yang memiliki kekonduksian elektriknya yang cemerlang dan kebolehannya memegang jumlah HBcAg yang tertinggi berbanding grafin oksida (GO) dan grafin oksida terturun (rGO-en). Penghasilan rGO-en-AuNPs ini telah mengguna pakai etilenadiamina sebagai agen penurun GO, dan juga bertindak sebagai agen untuk memegang nanopartikel emas di tempatnya di atas rGO. Keempat-empat jenis pencirian seperti mikroskop imbasan pancaran medan elektron (FESEM), belauan sinar-X (XRD), inframerah transformasian Fourier (FTIR), dan spektroskopi Raman telah mengesahkan bahawa GO telah diturunkan dan AuNPs telah berjaya digabungkan ke atas rGO-en. Kemudiannya, HBcAg yang telah dinyahgerakkan di atas rGO-en-AuNPs, yang telah terpasang di atas elektrod skrin bercetak (SPE) telah dibenarkan untuk menjalani pengesanan impedimetrik anti-HBcAg dengan kepekatan berjulat daripada 3.91 ng mL^{-1} to 125.00 ng mL^{-1} dengan antibodi yang menentang estradiol (anti-estradiol) dan albumin serum bovin (BSA) sebagai pengganggu. Setelah pengesanan anti-HBcAg di dalam sampel larutan penampan dengan had pengesanan 3.80 ng mL^{-1} pada 3 σ m^{-1} (persamaan regresi linear ΔR_{ct} =

0.0261[anti-HBcAg] + 6.421 (R^2 = 0.982)), pengesanan impedimetrik antibodi telah dijalankan di dalam campuran sampel serum manusia. Dalam kehadiran pengganggu, dan serum manusia, had pengesanan masing-masing telah didapati pada 3.88 ng mL^{-1} pada 3 σ m^{-1} (persamaan regresi linear ΔR_{ct} = 0.0315[anti-HBcAg] + 6.309 (R^2 = 0.971)), dan 5.60 ng mL^{-1} pada 3 σ m^{-1} (persamaan regresi linear ΔR_{ct} = 0.0345[anti-HBcAg] + 6.467 (R^2 = 0.961)). Tindak balas elektrokimia dalam semua pengesanan anti-HBcAg menunjukkan rintangan aliran elektron (R_{ct}) berkadar terus dengan kepekatan anti-HBcAg. Menariknya, tiada tindak balas silang dilihat apabila HBcAg yang telah dinyahgerakkan di atas rGO-en-AuNPs dibenarkan untuk bertindak balas dengan antibody yang tidak berkait. Peringkat pembangunan penderia biologi ini akan menjadi platfom asas untuk kajian akan datang dalam menghasilkan prototaip penderia imun anti-HBcAg yang mempunyai kesensitifan dan kekhususan yang lebih tinggi dan baik pada masa akan datang. Kerja-kerja pengecilan juga adalah munasabah untuk dijalankan berdasarkan kaedah elektrokimia yang telah dipilih.

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I certify that an Examination Committee has met on 8 February 2019 to conduct the final examination of Mohamad Farid bin Abd Muain on his degree thesis entitled "Development of Anti-Hepatitis B Virus Core Antigen Biosensor Using Gold Nanoparticle-Decorated Reduced 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 degree of Master of Science.

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LIST OF ABREVIATIONS

%	percent
°C	Degree celcius
λ	Wavelength
µg	Microgram
µm	Micrometer
µL	Microlitre
σ	Standard deviation
ΔR_{ct}	Changes in R_{ct}
-OH	Hydroxyl group
Anti-estradiol	Antibody against estradiol
Anti-HBcAg	Antibody against HBcAg
Anti-HBeAg	Antibody against HBeAg
Anti-HBsAg	Antibody against HBsAg
Ag	Silver
Ar	Argon
$AuCl_4^-$	Chloroauric anion
AuNPs	Gold nanoparticles
BSA	Bovine serum albumin
cm	Centimetre
C=C	Alkene
C=O	Carbonyl
C-O-C	Epoxy group
Cu	Copper

CV	Cyclic voltammetry
D/G	Intensity of D band to G band
dH_2O	Deionized water
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DPV	Differential pulse voltammetry
ELISA	Enzyme-linked immunosorbent assay
EI	Electrochemical immunosensor
EIS	Electrochemical impedance spectroscopy
en	Ethylenediamine
E_{pa}	Anodic peak potential
E_{pc}	Cathodic peak potential
e	Electron
$Fe(CN)_6^{3-}$	Ferricyanide anion
$Fe(CN)_6^{4-}$	Ferrocyanide anion
$Fe(CN)_6^{3-/4-}$	Redox reaction of ferricyanide and ferrocyanide ions
FESEM	Field emission scanning electron microscope
FTIR	Fourier transform infrared
g	Gram
GO	Graphene oxide
H_2SO_4	Sulfuric acid
$HAuCl_4$•3H2O	Chloroauric acid
HBcAg	Hepatitis B virus core antigen
HBeAg	Hepatitis B virus e antigen

HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HNO_3	Nitric acid
Hz	Hertz
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
K	Kelvin
kb	Kilobases
$K_3Fe(CN)_6$	Potassium ferricyanide
KCl	Potassium chloride
$KClO_3$	Potassium chlorate
kHz	Kilohertz
$KMnO_4$	Potassium permanganate
L-HBsAg	Large hepatitis B virus surface antigen
LOD	Limit of detection
M	Molar
m	Gradient of straight line
M-HBsAg	Middle hepatitis B virus surface antigen
mg	milligram
mL	Millilitre
mM	Millimolar
mV	Millivolt

NaH_2PO_4	Sodium dihydrogen phosphate
Na_2HPO_4	Disodium hydrogen phosphate
$NaNO_3$	Sodium nitrate
ng	Nanogram
Ni	Nickel
nm	Nanometre
OBI	Occult hepatitis B virus infection
P	Polymerase protein
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
ppm	Part per million
POC	Point of care
qPCR	Quantitative real-time PCR
RIA	Radioimmunoassay
R_{ct}	Resistant of charge transfer
RMS	Root mean square
rpm	Revolutions per minute
RSD	Relative standard deviation
Ru	Ruthenium
rGO	Reduced graphene oxide
rGO-en-AuNPs	Gold nanoparticles-decorated reduced graphene oxide
s	Second
S-HBsAg	Small hepatitis B virus surface antigen
SPE	Screen-printed electrode

Si	Silicon
SiO_2	Silicon dioxide
SiC	Silicon carbide
SWV	Square wave voltammetry
USB	Universal serial bus
V	Volt
w/v	Weight over volume
XRD	X-ray diffraction

CHAPTER 1

INTRODUCTION

Hepatitis B virus (HBV) comes from the family of *Hepadnaviridae* and the genus of *Orthohepadnaviridae* (Tan, 2016). Transmission of the virus can occur mainly via infected blood, and also by other body fluids such as saliva, sweat, semen and breast milk (Willey, Sherwood, & Woolverton, 2011). While humans are the only known natural host to HBV, this deadly virus can also infect other higher primates such as orangutans and chimpanzees (Shepard, Simard, Finelli, Fiore, & Bell, 2006; Tan, 2016).

HBV had infected more than one-third of the human population and had caused 1-2 million deaths annually by causing liver cirrhosis, liver cancer and other liver complications in humans (Michel & Tiollais, 2010; Tan, 2016). This highly contagious virus which is about 200 times more infectious than human immunodeficiency virus (HIV) had raised a serious threat to human health worldwide, particularly in South East Asia, China, and Africa. More than 8 % of the population were chronically infected by HBV (Tan, 2016). Vertical and perinatal transmission from mother to babies can also occur resulting in 90 % of the children chronically infected if the infection occurred in the first years of life (Michel & Tiollais, 2010).

In Malaysia alone, HBV infection constitute approximately 75 % out of all hepatitis infections where chronic HBV infection has contributed for 80 % of hepatocellular carcinoma cases (Raihan, 2016). Due to the commencement of universal vaccination of infants starting from 1989 by the Government of Malaysia, the incident cases of HBV had reduced from 1990 to 1997. In 1998, the HBV infection increased to nearly 25 cases for each of 100 000 populations due to the inclusion of cases for mandatory screening for all foreign workers who arrived in Malaysia. However, the decrement of reported cases after the year of 2000 started to rise again since 2010 due to the law implemented by the government where all HBV infection cases should be reported to the hospitals (Raihan, 2016).

The most commonly employed HBV screening tests are the detection of HBV DNA by polymerase chain reaction (PCR) and sensing the presence of HBsAg in the patient blood (Badrawy & Bakry, 2013; Krajden, McNabb, & Petric, 2005; Willey et al., 2011). However, these methods are insufficient as a single test for occult hepatitis B virus infection (OBI) patient screening as OBI patients may possess only HBV DNA but no detectable HBsAg. So, ultrasensitive PCR amplification with the limit of detection of 10 copies of viral DNA for each reaction need to be done. Despite being less cost effective and time-consuming, it may be the best option for detecting HBV in OBI patients (Hatzakis, Magiorkinis, & Haida, 2006). Interestingly, antibody against HBcAg (anti-HBcAg) had emerged as a convincing serological marker for detection of OBI, besides being a marker

to denote that the patient had been infected by HBV at any time in their lives (Cabral, Lima, Moura, & Dutra, 2015; Yano et al., 2003).

The development of electrochemical immunosensors for the application in various fields such as agriculture, industrial and environmental monitoring, food safety, biomedicine, development and screening of drugs, forensic analysis, prevention and control of epidemic disease as well as point-of-care (POC) diagnostic had received extensive advancement, but the main challenge faced by these methods is achieving the lowest limit of detection (LOD) (Lim & Ahmed, 2016; Wan, Su, Zhu, Liu, & Fan, 2013). Various strategies and architectures in the development of electrochemical immunosensors including enzyme-labelled immunosensor (Pei et al., 2013), quantum dot- and metal nanoparticle-labelled immunosensor (Z. P. Chen et al., 2007; Lai, Wang, Wu, Ju, & Yan, 2012; Lim, Yoshikawa, Tamiya, Yasin, & Ahmed, 2014), capacitive (S. Li et al., 2014) and impedimetric (Ionescu et al., 2010) immunosensor, as well as magnetoimmunosensors (de la Escosura-Muñiz et al., 2010) had been demonstrated.

This study demonstrated the synthesis of gold nanoparticles-decorated reduced graphene oxide (rGO-en-AuNPs) nanocomposite and simple immobilization of HBcAg onto the nanocomposite as bioreceptor for the sensing of anti-HBcAg antibody. Since this study uses the electrochemical approach to sense the presence of anti-HBcAg, each functionalized screen-printed electrode was analyzed through cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) upon each modification done to it. The combination of immunoaffinity and electrochemical concepts has been demonstrated as a promising way to develop anti-HBcAg biosensor with a simple design, yet exhibit high sensitivity and specificity, fast response with minimal interferences.

The objectives of this research were as follow:

- To detect anti-HBcAg by using the HBcAg-immobilized rGO-en-AuNPs
    - To functionalize reduced graphene oxide with antigen/protein-based bioreceptor i.e. Hepatitis B virus core antigen (HBcAg)
    - To investigate the electrochemical response upon each modification done, from bare electrode, HBcAg and BSA immobilization, up to anti-HBcAg detection
    - To perform sensor testing in spiked buffer and human serum sample
        - Detection of anti-HBcAg on HBcAg-functionalized electrode
        - Determining the LOD
        - Evaluating specificity and selectivity of immunosensor

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