UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF A BIO-NANOGATE-BASED ELECTROCHEMICAL IMMUNOSENSING STRATEGY FOR THE DETECTION OF ANTI-HEPATITIS B SURFACE ANTIGEN ANTIBODY

NOOR SYAMILA BINTI OTHMAN

FBSB 2022 12

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By

NOOR SYAMILA BINTI OTHMAN

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

March 2022

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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

An area requiring real-time analysis is the diagnosis of infectious disease and monitoring of vaccination efficiency against the disease to determine immunity level particularly among high risk group including immunocompromised patients against infectious diseases in screening for immunization program including hepatitis B virus (HBV). However, the conventional methods require laborious work and tedious fabrication of sensing platforms which limit the efficiency in upscaling the screening tests. These further impede the analysis of large cohort of clinical samples. Therefore, an effort is needed in order to improve the outcome of this lab-based technology. Generally, a bio-nanogate system involves the use of synthetic or natural molecules as a 'gate' towards bioreceptors and ideally, the gating mechanism should respond only upon the presence of external stimuli i.e. targeted analytes in a nanoscale dimension. The versatility of polyamidoamine (PAMAM) dendrimers to form conjugates with proteins can be utilized to form a bio-nanogate. PAMAM interaction with protein bioreceptor and the ability of a bio-nanogate-based immunosensing strategy in detecting an antibody i.e. anti-hepatitis B surface antigen (anti-HBsAg) antibody electrochemically were of interest in this study.

An antigenic determinant (aD) region of HBV fused with maltose binding protein (MBP-aD) was synthesized to form a specific bioreceptor for anti-HBsAg antibody in the bio-nanogate system. The bio-nanogate interaction was further analysed for its binding affinity, thermal stability, and thermodynamic analysis. Following that, a proof of concept utilizing displacement immunosensing strategy was conducted electrochemically, where the MBP-aD was immobilized on the screen-printed carbon electrode (SPCE) platform, and further sandwiched with electroconductive PAMAM encapsulated gold nanoparticles (PAMAM-Au), forming the 'gate'.

PAMAM-Au here also functions as a monitoring agent capable of generating a signal response upon a displacement event in the presence of anti-HBsAg antibody in differential pulse voltammetry (DPV) analysis. Finally, the PAMAM-Au displacement efficiency was further improved via implementation of acoustic mixing on modified SPCE platform coupled with piezoelectric transducer.

The synthesized MBP-aD was confirmed with western blotting technique. The interaction study revealed that the interaction of MBP-aD with anti-HBsAg antibody has a higher thermal stability and binding affinity (K_A = 1.6 x10^{-5} $Lmol^{-1}$) as compared to its interaction with PAMAM (K_A = 2.9 x 10^{-6} $Lmol^{-1}$). Thermodynamic parameters also demonstrated that the bio-nanogate components interact through van der Waals and hydrogen bonding. Based on the interaction study, it was hypothesized that the interactions among the bio-nanogate components may be able to be manipulated at the nanoscale level for the detection of anti-HBsAg antibody. Under optimal conditions, the hypothesis was proven that the high specificity of anti-HBsAg antibody towards MBP-aD displaced PAMAM-Au, in a range of 1mIU/mL to 1000 mIU/mL with a detection limit (LOD) of 2.5 mIU/mL. The results also showed high specificity and selectivity of the immunosensor platform in detecting anti-HBsAg antibody both in spiked buffer and human serum samples. Furthermore, the incubation/reaction time for detecting anti-HBsAg antibody has been reduced from an initial incubation time of 20 min to 8 min via the improvement of PAMAM-Au displacement efficiency under acoustic streaming effect. The newly developed immunosensor platform utilizing the manipulation of lower interaction between PAMAM-Au (gate) and the candidate bioreceptor (anchor) would ultimately eliminate the need of having specifically designed and labelled analogues which has been commonly used in displacement-based immunoassays.

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

PEMBANGUNAN STRATEGI PENDERIAAN IMUNO ELEKTROKIMIA BERDASARKAN GET NANO BIO UNTUK PENGESANAN ANTIBODI BAGI ANTIGEN PERMUKAAN VIRUS HEPATITIS B

Oleh

NOOR SYAMILA BINTI OTHMAN

Mac 2022

Pengerusi: Asilah binti Ahmad Tajudin, PhD
Fakulti: Bioteknologi dan Sains Biomolekul

Bidang yang memerlukan analisis waktu nyata adalah diagnosis penyakit berjangkit dan pemantauan kecekapan vaksinasi terhadap penyakit untuk menentukan kadar imuniti terutamanya golongan berisiko tinggi dan pesakit terimunokompromi terhadap penyakit berjangkit di dalam saringan program imunisasi termasuklah terhadap virus hepatitis B (HBV). Namun, kaedah konvensional yang memerlukan gerak kerja makmal yang rumit dan pembuatan platform pengesanan yang memakan masa mengurangi kecekapan dalam meningkatkan ujian saringan. Ini seterusnya membatasi analisis kohort sampel klinikal yang besar. Oleh itu, usaha diperlukan untuk meningkatkan hasil teknologi berdasarkan makmal ini. Secara amnya, sistem get nano bio melibatkan molekul yang disintesis atau molekul semula jadi sebagai 'pintu pagar' terhadap reseptor bio dan secara idealnya, mekanisme pintu pagar harus bertindak balas hanya apabila terdapat rangsangan luaran, iaitu analit yang disasarkan dalam dimensi skala nano. Keserbagunaan dendrimer poliamidoamina (PAMAM) untuk membentuk konjugat dengan protein dapat digunakan dalam membentuk get nano bio. Interaksi PAMAM dengan reseptor protein dan kemampuan strategi penderiaan imuno berasaskan get nano bio dalam mengesan antibodi iaitu antibodi anti-HBsAg, secara elektrokimia menarik minat kajian ini.

Bahagian penentuan antigen (aD) yang dilakurkan dengan protein penambat maltosa (MBP-aD) telah disintesis untuk membentuk reseptor bio khusus kepada antibodi anti-HBsAg dalam sistem get nano bio. Interaksi get nano bio dianalisis lebih lanjut untuk afiniti pengikat, kestabilan terma, dan analisis termodinamik. Berikutan itu, bukti konsep yang menggunakan strategi pengalihan dalam penderiaan imuno dilakukan secara elektrokimia, di mana MBP-aD dipegunkan di atas

platform skrin bercetak elektrod karbon (SPCE), dan selanjutnya dilapisi dengan nanopartikel emas berkapsul PAMAM (PAMAM-Au) yang elektrokonduktif untuk membentuk 'pintu pagar'. PAMAM-Au di sini juga berfungsi sebagai agen pemantauan yang mampu menghasilkan isyarat tindak balas apabila berlaku kejadian peralihan dengan kehadiran antibodi anti-HBsAg menerusi analisis voltammetri nadi pembezaan (DPV). Akhirnya, kecekapan peralihan PAMAM-Au ditingkatkan lagi melalui pelaksanaan pencampuran akustik pada platform SPCE yang digunakan bersama dengan transduser piezoelektrik.

MBP-aD yang disintesis disahkan dengan kaedah 'western blotting'. Kajian interaksi menunjukkan bahawa interaksi MBP-aD dengan antibodi anti-HBsAg mempunyai kestabilan terma dan afiniti pengikatan yang lebih tinggi (K_A = 1.6 x10^{-5} Lmol^{-1}) berbanding interaksinya dengan PAMAM (K_A = 2.9 x 10^{-6} Lmol^{-1}). Parameter termodinamik menunjukkan bahawa komponen get nano bio berinteraksi melalui ikatan van der Waals dan hidrogen. Berdasarkan kajian interaksi, dihipotesiskan bahawa interaksi antara komponen get nano bio dapat dimanipulasi pada skala nano untuk pengesanan antibodi anti-HBsAg. Dalam keadaan optimum, hipotesis terbukti bahawa kekhususan tinggi antibodi anti-HBsAg terhadap MBP-aD dapat mengalihkan PAMAM-Au, dalam julat 1mIU / mL hingga 1000 mIU / mL dengan had pengesanan (LOD) 2.5 mIU / mL. Hasilnya juga menunjukkan kekhususan dan selektiviti tinggi platform imunosensor dalam mengesan antibodi anti-HBsAg dalam sampel larutan penimbal dan sampel serum manusia. Masa inkubasi / reaksi untuk mengesan antibodi anti-HBsAg juga dapat dikurangkan dari masa inkubasi awal 20 min menjadi 8 min melalui peningkatan kecekapan peralihan PAMAM-Au di bawah kesan penstriman akustik. Platform imunosensor yang baru dibangunkan menggunakan manipulasi interaksi rendah antara PAMAM-Au (pintu pagar) dan calon reseptor bio (jangkar) ini akan menyingkirkan keperluan dalam menggunakan analog yang direka dan dilabel secara khusus yang biasa digunakan dalam imunoasai berdasarkan peralihan.

ACKNOWLEDGEMENTS

Alhamdulillah, all praise and glory to Allah the Almighty, by whose Grace and Blessings. His guidance gave me the courage and patient to complete this postgraduate journey. Peace and blessing of Allah be upon the Prophet Muhammad (PBUH).

There are many people whom I wanted to thank wholeheartedly for their help had been a wonderful support for me throughout this journey. There has been a good atmosphere, where people are considerate and thoughtful. We can openly discuss new ideas and this has been crucial for me to succeed and accomplish this PhD degree.

My sincere gratitude towards my supervisor, Assoc. Prof. Dr. Asilah Ahmad Tajudin, who was the one actually believed in my 'innocent' rough idea and extended her view on how it can be done. It has been very important for me to have a dependable supervisor with whom I can have-in-depth discussions on how the biosensor actually works with. I can say we have a great 'student-teacher' and 'mother-daughter' -like relationship as she accommodates me with every help she can give and get towards myself and the project, emotionally, physically and financially. Most of all, I greatly appreciate the confidence she put and allowed me to develop and pursue new ideas and influence the direction of this research.

I also want to thank my supervisory committee members; Assoc. Prof. Dr. Amir Syahir Amir Hamzah, Prof. Dr. Tan Wen Siang, Assoc. Prof. Dr. Yusran Sulaiman and Assoc. Prof. Dr. Haslina Ahmad for creating a good research environment and critically discussing on the length of this project. Over the years, we had several great research discussions in integrating the biology, chemistry and physics parts to develop this new concept. Not to forget, my Japanese sensei in Kyutech, Assoc. Prof. Dr. Shinya Ikeno who has endlessly support me in the earlier part of this project. His positive attitude and kindness towards his students have been very motivating.

Next on the thank you-list are the nanobiotechnology research group members who were continuously giving fruitful discussions throughout the study and also the member of EMTech for their tremendous helps, hospitality and kindness which were beyond compared. Most of all, special dedication for my husband, Muhammad Syafiq, whom always been my comfort and happiness since the beginning of this journey. I also thanked my lifelong supporter; ummi, ayah, atok and my siblings for being very supportive and always showing a great interest in my study. Last but not least, I would like to give my gratitude to all of my best friends, new local and international friends that I met during my research attachment in Japan and to all the colleagues who have helped to keep my sane and stay put in this entire PhD study.

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:

Asilah binti Ahmad Tajudin, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Tan Wen Siang, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Amir Syahir bin Amir Hamzah, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Haslina binti Ahmad, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

Yusran bin Sulaiman, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

_____
ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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%	Percentage
[*a*]	Concentration of analyte
°C	Degree celsius
μ	Viscosity
μA	Microampere
μL	Microliter
μm	Micrometer
μM	micromolar
3D	Three-dimensional
A_{600nm}	Optical density at wavelength 600 nanometer
aa	amino acid
aD	antigenic determinant
AFM	Atomic force microscopy
Ag	silver
Anti-HBcAg	Anti-hepatitis B core antigen antibody
Anti-HBsAg	Anti-hepatitis B surface antigen antibody
AP	Alkaline phosphatase
APS	Ammonium persulfate
Au	Gold
$AuCl_4^-$	Tetrachloroaurate ion
B	Signal intensity
b	y-intercept
BCIP-NBT	5-Bromo-4-chloro-3-indolyl phosphate – nitro blue tetrazolium

BSA	Bovine serum albumin
C	Carbon
C=O	Carbonyl group
$CaCl_2$	Calcium chloride
CIMA	Chemiluminescence assay
C—N	Carbon-nitrogen bond
CV	Cyclic voltammetry
dH_2O	Deionized water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DPV	Differential pulse voltammetry
DTGS	Deuterated Triglycine sulfate
E	potential
E. coli	*Escherichia coli*
EI	Electrochemical immunosensor
EIS	Electrochemical impedance spectroscopy
f	Frequency
FESEM-EDX	field emission scanning electron microscopy with Energy Dispersive X-ray spectroscopy
F_{max}	Maximal fluorescence
F_{min}	Minimal fluorescence
FTIR	Fourier-transform infrared spectroscopy
g	Gram
H^+	Hydrogen ion
H_2PO_4	Dihydrogen phosphate

HBsAg	Hepatitis B surface antigen
hr	Hour
Hz	Hertz
I	current
IgG	Immunoglobulin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
J	Joule
K_A	Binding adsorption constant
K	Kelvin
$K_3Fe(CN)_6$	potassium hexacyanoferrate (III)
$K_4[Fe(CN)_6]*3H_2O$	potassium hexacyanorerrate (II) trihydrate
KBr / Ge	Potassium bromide / germanium
kDa	kilodaltons
kHz	kilo-Hertz
KPL	Milk diluent / blocking solution
L	Litre
LB	Luria-Bertani
LOD	Limit of detection
LSV	Linear sweep voltammetry
M	Molar
m	Slope
mA	Milliampere
MBP	Maltose binding protein
MBP-aD	Maltose binding protein fused with antigenic determinant

MCS	Multiple cloning sites
$MgCl_2$	Magnesium chloride
MHz	Mega-Hertz
min	Minute
mIU	milli-international unit
MOPS	3-(N-morpholino)propanesulfonic acid
N	Number of available sites
Na_2HPO_4	disodium hydrogen phosphate
NaCl	Sodium chloride
NaH_2PO_4	sodium dihydrogen phosphate
Nano-DSF	Nano-differential scanning fluorometry
ng	Nanogram
N—H	Nitrogen-nitrogen bond
NH_3^+	Ammoniumyl
nm	Nanometer
NPs	Nanoparticles
O	Oxygen
P	Fluorescence intensity
PAMAM	Polyamidoamine dendrimer
PBS	Phosphate buffered solution
R	Resistance
RbCl	Rubidium chloride
R_{ct}	Resistant of charge transfer
RMS	Root mean square
rpm	Revolutions per minute

RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	Second
SEM	Scanning electron microscope
SPCE	Screen-printed carbon electrode
TBS	Tris-buffered saline
TBST	Tris-buffered saline with 0.1% TWEEN® 20 Detergent
TFBI	Transformation buffer I
TFBII	Transformation buffer II
T_i	Inflection temperature
Tris-HCl	Tris hydrochloride
Trp	Tryptophan
UV-vis	Ultraviolet-visible spectroscopy
V	Volt
Vpp	Voltage peak-to-peak
W	Watt
Y	Fluorescence ratio
β	Beta
ΔR_{ct}	Changes of resistant of charge transfer
$\Delta_A G°$	standard Gibbs free energy of associated protein-polymer
$\Delta_A H°$	enthalpy
$\Delta_A S°$	Entropy
ζ-potential	Zeta potential

ρ	density
σ	Viscous boundary thickness

CHAPTER 1

INTRODUCTION

The emergence and re-emergence of viral infectious diseases including hepatitis B virus, (HBV), E-bola virus, immunodeficiency virus (HIV), and the recent severe acute respiratory syndrome (SARS)-associated coronavirus 2 (SARS-CoV-2) that caused COVID-19 have caused major public health concerns worldwide. The general transmission factor involving human as a host risks in viral infection attacking vital organs and this can cause acute or chronic diseases particularly in immunocompromised individuals. The protection against viral infection can be obtained through natural or acquired immunity such as vaccination, where it can trigger the immune response to recognize and fight against the viral-causing diseases.

Vaccination efficiency is typically assessed by determining the level of immunity developed in the body particularly among high risk group such as healthcare and foreign workers, immunocompromised patients and antenatal mothers during their visit in primary healthcare centres (Tam et al., 2017; Roupa et al., 2019). Monitoring antibodies against viral antigens is critical for maintaining immunity, especially in vaccinated individuals and immunocompromised patients (Grzegorzewska, 2012; Tan & Ho, 2014; Yong et al., 2015). Antibody screening tests frequently involve the detection of virus-specific antibodies through the use of conventional methods such as enzyme-linked immunosorbent assay (ELISA) and western blot analysis. Immunodetection assays identify and quantify specific targeted antibodies. The results of this detection are required in diagnostic and treatment procedures for viral infections. However, these conventional methods require laborious work and are time consuming. These limit the efficiency of upscaling the screening tests and further impede the analysis of large cohorts of clinical samples (Poiteau et al., 2017; Tam et al., 2017). Rapid diagnostic tests (RDTs) on the other hand, were developed to assist clinicians in preliminary screening and predicting the risk of reinfection in the infected individuals, particularly in remote areas. This adds benefit in disease surveillance and epidemiologic research. The most well-known RDT to date is probably the lateral flow tests, however they are limited in sensitivity, expensive, require multistep fabrication, and have reproducible issues due to the limitation of fade dyes in colorimetric-based sensor, which may lead to false negative results (Sher et al., 2017). Henceforth, more research is required to overcome the current limitations and develop reliable antibody detection systems in the future.

There are various advantages of using electrochemical-immunosensors for infectious disease markers. Among the benefits are ease of handling, robustness, portability, shorter detection times and relatively inexpensive analytical systems, all of which contribute to the development of point-of-care tests (POCTs) (Kaushik et al., 2018; Layqah & Eissa, 2019; Fabiani et al., 2021; Eissa & Zourob, 2021). Among them is the displacement-based electrochemical immunosensor, which uses the competitive-heterogenous-like immunoassay concept to measure signals from the immunocomplex formation via current or impedance change (Tang et al., 2018; Safarian et al., 2021). The displacement of non-specific binding of analyte analogue from bound bioreceptors in the presence of free targeted analyte offers a simple fabrication such as reagentless and washing-free steps. However, the high steric effect of analogue makes displacement difficult and occurred only after a longer incubation time (Khor et al., 2013; Lu et al., 2016; Avella-Oliver et al., 2018).

Hepatitis B virus (HBV) is a DNA virus that infects the human liver and causes both acute and chronic liver diseases. It affects approximately two billion people worldwide, of which around 257 million people suffering from chronic HBV infections (Papastergious et al., 2015; Huzair &Sturdy, 2017; World Health Organization, 2019). A common serological marker of HBV, the anti-hepatitis B surface antigen antibody (anti-HBsAg antibody) indicates the immune status and the viral clearance in infected individuals. Such antibody is developed upon natural exposure towards HBV or through immunization with HBsAg (Pondé, 2011). To date, the majority of anti-HBsAg antibody detection is done using the conventional immunoassay, which has sensitivity issues when detecting low antibody titres and requires longer incubation time, limiting the turnaround analysis in time (Grzegorzewska, 2012; Tan & Ho, 2014; Yong et al., 2015). Meanwhile, RDT demonstrated low sensitivity in detecting low antibody titres and was only reliable in the presence of positive test (Bottero et al., 2013; Chevaliez & Pawlotsky, 2018). As a result, in this study, anti-HBsAg antibody was chosen as a model analyte marker for the formation of the current antibody detection system.

Recently, the fabrication of nanogate/nanoswitch has attracted the interest of researchers, particularly in developing biosensors and drug carriers, as the ‘gating’ mechanism. This ‘gating’ mechanism which can be in the form of synthesized polymers or biomolecules may possess the designated recognition sites that can respond selectively to external stimuli and is able to regulate signal release from a solid support (Tam et al., 2013; Bilalis et al., 2016; Huang & Szleifer, 2017; Luo et al., 2019; Yang et al., 2020). This mechanism allows small molecules to be detected in a sensitive manner (Lee et al., 2016; Vivero-Escoto et al., 2017). Several studies have shown that the use of nanogate systems in conjunction with the use of porous materials can transiently hold the signalling molecules that are engulfed within the sensor surface (Wang et al., 2015; Huang & Szleifer, 2017). Apart from that, the ability of DNA and aptamer-based molecules to form a ‘gate’ within a bio-nanogate system that relies on low interactions of

electrostatic, hydrophobic and hydrogen bonding also demonstrated selectivity towards targeting biological components (Wang et al., 2015). However, the aptamer-based bio-nanogate system's time-consuming fabrication and the requirement of multi-reagent steps limit the analysis turnaround time. The manipulation of 'gating' mechanism could be beneficial in improving the displacement-based electrochemical immunosensing strategy particularly for antibody detection. An appropriate component to function as the 'gate' in a bio-nanogate system is crucial for this purpose to ensure good control over its operation.

The sensitivity of the biosensors can also be improved by monitoring the small detectable current changes that may have occurred on the electrode. The current changes can be obtained when displacement-based immunosensor applies the non-specific binding components to react with the bioreceptors, and it will then be displaced with the presence of a specific targeted analyte (Zhang & Ding, 2016; Nadhakumar et al., 2018; Roy & Sharan. 2019). The displacement occurrences can be elevated by incorporating the acoustic streaming forces, which can manipulate and enhance the mass transport of surface-attached biomolecules by causing streaming-induced drag forces and swirling motion in the interacted media. Acoustic streaming has been reported to aid in many applications, including acceleration of DNA-hybridization assay, particle concentration, manipulation of micro/nanoparticles and fluid mixing within microfluidic channels (Liu et al., 2003; Daniels et al., 2011; Collins et al., 2016; Cui et al., 2016; Collins et al., 2017).

Therefore, this study was carried out to develop an immunosensor which incorporates a bio-nanogate mechanism to detect anti-HBsAg antibody on electrode surface, while utilizing electrochemical signalling, which in turn is crucial for the development of a POCT tool. The interaction of bio-nanogate key component biomolecules of synthesized maltose binding protein (MBP) harboring antigenic determinant (aD) region of HBsAg (MBP-aD) as a bioreceptor, PAMAM (the gate) and anti-HBsAg antibody was investigated. This is followed by the 'proof-of-concept' of the bio-nanogate-based immunosensing strategy employing the concept of displacement immunosensing via nonspecific-binding of PAMAM-Au to form weak interactions, which can act as a 'gate' towards its 'anchor' of potential bioreceptors. The presence of PAMAM-Au is also useful as a signal label for the monitoring of the oxidation peak changes upon displacement of the 'gate' from the 'anchor' in detecting the analyte using differential pulse voltammetry (DPV). The displacement efficiency of PAMAM-Au can be improved via acoustic streaming forces and reduces the standing incubation/reaction time of the developed immunosensor in targeting anti-HBsAg antibody.

1.1 Hypotheses

1. Bio-nanogate-based immunosensor utilizing displacement immunosensing strategy may improve rapidity, simplicity and sensitivity in targeting anti-HBsAg antibody, electrochemically.
2. The presence of the aD region as bioreceptor can enhance the specificity and accuracy of the sensing platform.
3. PAMAM will have lower binding interaction with the MBP-aD to form bio-nanogate.
4. The presence of anti-HBsAg antibody which has a higher affinity towards MBP-aD will displace PAMAM in the anti-HBsAg antibody detection system.
5. The synthesized PAMAM-Au can play a major role as a monitoring agent to monitor the displacement occurrences upon detection.
6. The incubation time for detecting anti-HBsAg antibody will be reduced via improvement of PAMAM-Au displacement efficiency under acoustic streaming effect.

1.2 General Objective

To develop an immunosensor which manipulates the interaction among bio-nanogate components at nanoscale level to improve simplicity, rapidity and sensitivity in targeting anti-HBsAg utilizing displacement immunosensing strategy, electrochemically

1.3 Specific Objectives

1. To synthesize the bioreceptor of maltose binding protein harbouring the antigenic determinant (MBP-aD) targeting the anti-HBsAg antibody in *Escherichia coli* expression system.
2. To analyze the interaction of bio-nanogate key components of MBP-aD, PAMAM and anti-HBsAg antibody using binding, thermal stability and thermodynamic analyses.
3. To synthesize and characterize the PAMAM encapsulated gold nanoparticles (PAMAM-Au)
4. To develop the bio-nanogate-based electrochemical immunosensor via non-specific binding of PAMAM-Au and MBP-aD for detection of anti-HBsAg antibody.
5. To improve the PAMAM-Au displacement efficiency via acoustic streaming forces for detecting anti-HBsAg antibody.

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