AN INTEGRATED RGO-PAMAM THIN FILM WITH SPR TECHNIQUE FOR DENGUE VIRUS DETECTION

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

AN INTEGRATED RGO-PAMAM THIN FILM WITH SPR TECHNIQUE FOR DENGUE VIRUS DETECTION

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January 2020

Chairman : Assoc. Prof. Yap Wing Fen, PhD
Faculty : Institute of Advanced Technology

The growing demand for early detection of dengue virus (DENV) has contributed to integrated thin films with surface plasmon resonance (SPR) technique, i.e. Au/Fe-NCC-CTAB/IgM, Au/CdSQDs-RGO/IgM, Au/CdSQDs-PAMAM/IgM, and Au/DSU/RGO-PAMAM/IgM. Their sensing performances such as sensitivity, binding affinity, full-width-half-maximum (FWHM), detection accuracy (DA), and figure-of-merit (FOM) were analysed.

The first developed Au/Fe-NCC-CTAB/IgM sensor film yielded the sensitivity of 27.051 °/nM, binding affinity of 0.862 TM^{-1}, and high performances of DA and FOM on detection of 0.001 nM of DENV E-proteins. For Au/CdSQDs-RGO/IgM sensor film, the detection performance showed the sensitivity and binding affinity values of 5.496 °/nM and 0.775 TM^{-1}, respectively. The results also showed that the sensor film had the highest DA and FOM values when detecting 0.001 nM of DENV E-proteins. The third developed Au/CdSQDs-PAMAM/IgM sensor film showed the sensitivity of 7.577 °/nM, binding affinity of 2.696 TM^{-1}, and high performances of DA and FOM on detection of 0.001 nM of DENV E-proteins. The final development of SPR sensor integrated Au/DSU/RGO-PAMAM/IgM sensor film resulted in the sensitivity of 10.939 °/nM, binding affinity of 9.783 TM^{-1}, and high performances of DA and FOM on detection of 0.0001 nM of DENV E-proteins. Structural investigations using Raman spectroscopy, Ultraviolet-visible near infrared (UV-Vis-NIR) spectroscopy, and Atomic force microscopy (AFM) have confirmed the presence of the conjugate DENV E-proteins-antibodies-nanocomposite layer.

Most importantly, by comparing the performances of the sensor films described above, the results found that the Au/DSU/RGO-PAMAM/IgM is a very effective sensor film because it has the lowest quantitation limit and strongest binding affinity. Despite its low sensitivity, the sensitivity of detection was further enhanced by optimizing the fabrication of Au/DSU/RGO-PAMAM/IgM sensor film. The excellent sensitivity and binding affinity

constant obtained were 333.896 °/nM and 9.345 TM^{-1}, respectively. High performance of DA, FOM, selectivity, stability, reproducibility, and spike-recovery were achieved at DENV E-proteins concentration of 0.00008 nM (0.8 fM), indicating the best concentration limit obtained so far. In addition, this sensor film demonstrated a real-time DENV E-proteins detection within 8 minutes. Studies from structural measurements have successfully showed the presence of functional groups, absorption band shifts, and increased in surface roughness values from 1.56 nm to 3.19 nm due to the binding event of sensing layer to DENV E-proteins.

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

FILEM NIPIS RGO-PAMAM BERSEPADU DENGAN TEKNIK SPR UNTUK MENGESAN VIRUS DENGGI

Oleh

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Permintaan yang semakin meningkat untuk mengesan virus denggi di peringkat awal telah menyumbang kepada filem nipis bersepadu dengan teknik resonan plasmon permukaan (SPR) iaitu *Au/Fe-NCC-CTAB/IgM, Au/CdSQDs-RGO/IgM, Au/CdSQDs-PAMAM/IgM, dan Au/DSU/RGO-PAMAM/IgM.* Prestasi penderiaan seperti kepekaan, pertalian pengikatan, lebar penuh pada separuh maksimum (FWHM), ketepatan pengesanan (DA), dan angka merit (FOM) telah dianalisis.

Filem penderiaan yang pertama iaitu *Au/Fe-NCC-CTAB/IgM* telah menghasilkan kepekaan sebanyak 27.051 °/nM, pertalian pengikatan sebanyak 0.862 TM^{-1}, dan prestasi DA dan FOM yang tinggi pada pengesanan 0.001 nM virus denggi *E-proteins*. Untuk filem penderiaan *Au/CdSQDs-RGO/IgM*, prestasi penderiaan menunjukkan kepekaan dan pertalian pengikatan sebanyak 5.496 °/nM dan 0.775 TM^{-1}. Keputusan juga menunjukkan bahawa filem penderiaan mempunyai nilai tertinggi DA dan FOM apabila mengesan 0.001 nM virus denggi *E-proteins*. Filem penderiaan yang ketiga iaitu *Au/CdSQDs-PAMAM/IgM* telah menunjukkan kepekaan sebanyak 7.577 °/nM, pertalian pengikatan sebanyak 2.696 TM^{-1}, dan prestasi DA dan FOM yang tinggi pada pengesanan 0.001 nM virus denggi *E-proteins*. Pembangunan terakhir penderian SPR yang digabungkan bersama *Au/DSU/RGO-PAMAM/IgM* telah menghasilkan kepekaan sebanyak 10.939 °/nM, pertalian pengikatan sebanyak 9.783 TM^{-1}, dan prestasi DA dan FOM yang tinggi pada pengesanan 0.0001 nM virus denggi *E-proteins*. Penyiasatan struktur menggunakan spektroskopi Raman, spektroskopi inframerah (UV-Vis-NIR) yang dilihat oleh ultraungu dan mikroskopi kuasa atom (AFM) telah berjaya mengesahkan kehadiran konjugasi lapisan virus denggi *E-proteins*-antibodi-nanokomposit.

Yang paling penting, dengan membandingkan prestasi penderiaan yang dinyatakan di atas, keputusan mendapati bahawa *Au/DSU/RGO-PAMAM/IgM* merupakan filem penderiaan yang sangat cemerlang kerana mempunyai had minimum kuantifikasi dan pertalian pengikatan yang kuat. Walaupun mempunyai kepekaan yang sedikit rendah, kepekaan

pengesanan semakin dipertingkatkan dengan mengoptimumkan pembuatan filem penderiaan *Au/DSU/RGO-PAMAM/IgM*. Kehebatan kepekaan dan pertalian pengikatan yang didapati adalah sebanyak 333.896 °/nM dan 9.345 TM^{-1}. Prestasi tinggi DA, FOM, pemilihan, kestabilan, kebolehulangan, dan *spike-recovery* telah dicapai pada kepekatan virus denggi *E-proteins* 0.00008 nM (0.8 fM), menunjukkan had kepekatan terbaik diperolehi setakat ini. Selain itu, filem penderiaan ini telah menunjukkan pengesanan virus denggi *E-proteins* dalam masa nyata selama 8 minit. Kajian-kajian dari pengukuran struktur berjaya menunjukkan kehadiran kumpulan-kumpulan berfungsi, pergeseran jalur penyerapan, dan kenaikan nilai kekasaran permukaan dari 1.56 nm ke 3.19 nm disebabkan hubungan pengikatan lapisan penderian kepada virus denggi *E-proteins.*

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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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LIST OF ABBREVIATIONS

Fe-NCC-CTAB	Iron oxide-cellulose nanocrystals-hexadecyltrimethylammonium bromide
CdSQDs-RGO	Cadmium sulfide quantum dots-amine functionalized graphene oxide
CdSQDs-PAMAM	Cadmium sulfide quantum dots-polyamidoamine dendrimer
DSU/RGO-PAMAM	Dithiobis succinimidyl undecanoate/amine functionalized graphene oxide-polyamidoamine dendrimer
NS1	Non-structural 1
mAb/ IgM	Monoclonal antibody
ZIKV	Zika virus
DENV	Dengue virus
K_A	Association equilibrium constant
K_D	Dissociation equilibrium constant
k''	Imaginary part of wave vector
k'	Real part of wave vector
n	Refractive index
θ_{SPR}	SPR resonance angle
nM	Nanomolar
TM	Teramolar
pM	Picomolar

CHAPTER 1

INTRODUCTION

1.1 Dengue disease

Dengue disease is caused by dengue virus (DENV), a mosquito-borne flavivirus. The DENV continues to be an unmet public health concern, which dramatically growing in tropical and subtropical regions around the world (Cucunawangsih & Lugito, 2017). The important factors include the uncontrolled growth of urban population, deforestation processes, lack of vector control in dengue endemic areas, increased air travel, and inadequate public health care systems such as sewage and waste management have contributed to the severe increased of DENV (Higa, 2011; Cuello et al., 2018) . The fastest infections DENV into a human body is caused by the bite of a female mosquitoes *Aedes aegypti*. The DENV consists of a single-stranded positive sense ribonucleic acid (RNA) genome comprising approximately 1100 nucleotide base, where it is surrounded by viral envelope (Gebhard et al., 2011; Dias et al., 2013;). Translation of viral RNA produces a single large polyprotein that is subsequently cleaved into the formation of three structural proteins (capsid, membrane, envelope protein) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) (Kroschewski et al., 2009). The structural protein forms the coat of the virus and help delivering the RNA to target host cell. Meanwhile, the non-structural proteins organize the production of a new virus in the host cell (Watterson et al., 2012; Tuiskunen Bäck & Lundkvist, 2013; Chen et al., 2018; Silva et al., 2019). The DENV is classified into four antigenically serotypes, DENV 1, DENV 2, DENV 3 and DENV 4 (Alvarez et al., 2005; Gonçalves et al., 2012; Halsey et al., 2012; Tripathi et al., 2012; Qiu et al., 2018; Swaminathan & Khanna, 2019). A serotype is known as a group of viruses that classified together based on their antigens on the surface of the virus. Each serotype produces different symptoms that range from classical dengue fever to the more severe and fatal diseases (Krishnappa & Paramasivam, 2015). Among four serotypes, DENV 2 appears to be the most prevalent agents that have caused severe diseases involving systemic vascular leakage, haemorrhage and/or organ dysfunction (Pozo-Aguilar et al., 2014; Shivanthan et al., 2015; Chanthick et al., 2018). The infection from DENV will provide an immune response to the human body by producing a slow and low titer immunoglobulin M (IgM), followed by immunoglobulin G (IgG), and immunoglobulin A (IgA) antibody that are mainly specific to the dengue virus.

1.2 Envelope proteins of DENV

Envelope proteins or E-proteins are a glycoprotein that carry 53-60 kDA of molecular weight (Guardia et al., 2017; Yap et al., 2017; Zhang et al., 2017). They are protein dimers on the surface of the mature viral particle that play a vital role in the dengue lifecycle. Each viral particle has 180 monomers of E-protein that are organized into 90 tightly packed dimers, and each of E-protein monomers composed of three domains (DI, DII, and DIII). DI is a structurally central domain of the envelope protein which stabilizes the overall orientation of the protein, DII is thought to be involved in membrane fusion, and DIII is responsible for the initial cellular receptor binding and appears to be a main target of

neutralization antibodies (Wahala & Silva, 2011; Halstead & Thomas, 2018; Rey & Lok, 2018). The initiation of DENV infection occurs when E-proteins bind to host cell receptors that would lead to membrane fusion, and subsequently to gene mutation (Cruz-Oliveira et al., 2015; Yap et al., 2017; Kamil et al., 2018). Given their key role in host cell entry, the E-proteins are an important target that is very effective for research to develop vaccine candidates and antiviral therapeutics.

1.3 Surface plasmon resonance phenomenon

A plasmon is a quantum of plasma oscillation of the free electrons, and the free electron gas has a collective oscillation relative to the fixed positive ions in a metal. The surface plasmons are those plasmon with collective electron oscillations that are confined to dielectric-metal interface. It can be excited by electromagnetic radiation by matching the momentum of the incident photon to that surface plasmon or so-called surface plasmon resonance condition. The excited surface plasmon can propagate along the interface between metal and the dielectric or can be localized on metallic nanoparticles or nanostructures (Garcia, 2011; Abdulhalim, 2018). The induced electromagnetic field on metal surface decays exponentially into both media as a surface confined evanescent wave. Since the induced field is very sensitive to the variations of dielectric medium in proximity to a metal surface, a small change in the optical properties of the dielectric medium can be quantitatively analysed for the sensing purposes (Xu et al., 2011; Spada & Vegni, 2018).

Sensors based surface plasmon resonance phenomenon have received continuously growing attention from scientific community. It has become a central tool for characterizing and quantifying biomolecular interactions by allowing real time analysis of the binding events without the use of labelled molecule (Homola, 2008; Nguyen et al., 2015). Its label-free advantage has become a leading technology in the field of immune sensors, DNA sensors, proteomics and drug discovery (Bartollino et al., 2017).

1.4 Self-assembled monolayers

Controlling the properties of metal surfaces is a challenge that has attracted wide scientific, as well as technological interest. This is because the bare thin metal films tend to absorb adventitious organic materials readily because these adsorbates lower the free energy of the interface between the metal film and the ambient temperature (Adamson & Gast, 1997). These adsorbates can modify the interfacial properties and as well can influence the stability of metal nanostructures as they have a profound role as an electrically insulating film or as a physical or electrostatic barrier against aggregation. Surface coated with adventitious materials are, however, not well-defined because they do not present specific chemical functionalities and do not have reproducible physical properties such as conductivity, wettability, or corrosion resistance (Love et al., 2005).

One way to activate metal surfaces with versatile functions, especially to control the chemical or physical properties of the surfaces is by modifying them with well-ordered molecular films. Self-assembly is a method that has attracted significant interest in physics,

chemistry and biology, because it provides a convenient, flexible and a simple system of creating a highly ordered thin molecular film with tailored chemical and physical properties (Ye et al., 2011). In a self-assembly process, an ultra-thin molecular layer is formed by the adsorption of molecules from solution onto a solid surface (ColoradoJr & Lee, 2001; Ozin et al., 2009). Then, the adsorbates spontaneously arrange themselves until finally a completely ordered molecular monolayer is formed, a so-called self-assembled monolayer (SAM).

SAMs are organized layers of molecules which formed spontaneously on a metal surface. Such a SAM consisting of a head group (thiols, organosilanes, carboxylic acids), tail and functional end group (Figure 1.1). The most extensively used SAMs' headgroup is based on the absorption of thiol molecules on metal surfaces. The thiol headgroup is one of the functionalities that form a strong interaction with noble metals (Jadhav, 2011; Valášek et al., 2016; Casalini et al., 2017). Therefore, in this study, a gold was chosen as a preferred substrate compared to silver and copper as they cannot easily to be oxidized which can support the formation of SAMs. Formation of SAM are induced by the strong chemical reactions between the substrate and the head group, while the tail groups assemble away from the substrate surface (Schreiber, 2000). This interaction is considered a result of chemisorption that forced a thiorate molecule to adsorb commensurate with a gold lattice. Then, the tail-to-tail interaction of the molecules created by lateral interchain nonbonded interactions, such as by van der Waals, steric, repulsive and electrostatic forces, is strong enough to align the molecules parallel on the gold surface and create a crystalline film (Schönenberger et al., 1995) .

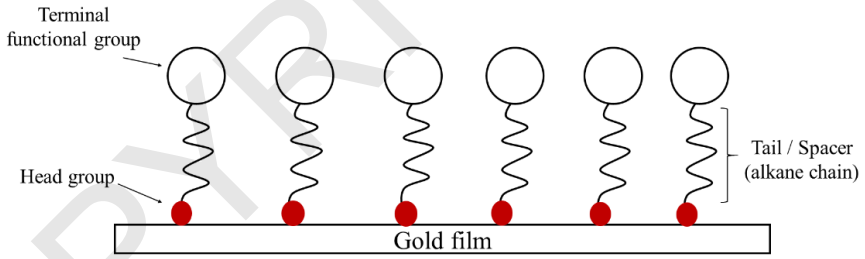

Figure 1.1: Schematic of self-assembled monolayer structure.

1.5 Problem statement

Over the past years, dengue non-structural 1 (NS1) antigen capture immunoassays have been identified and proven to be an effective tool in the diagnosis of dengue. However, detection towards NS1 antigen tends to be less sensitive in secondary dengue (DENV 2) infection than in primary dengue (DENV 1) infection (Kumarasamy, 2007; Bessoff et al., 2008; Wong et al., 2016), thus, it is beyond our goal to detect DENV 2. To overcome these barriers, it is of our interest to aim for early detection of DENV by employing the envelope (E) proteins of the DENV 2 as our determinant. The E-protein is one of the protein structures that was on the host virus itself compared to NS1, a metabolic product that induced by the virus infection after viremia phase. Therefore, it gives us a room to achieve early detection of DENV at the onset of infection by targeting DENV E-proteins.

Several standard tests have been developed for diagnosis of dengue virus infections. The diagnostic methods with application in routine analysis that normally used include enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), reverse transcriptase-polymerase chain reaction (RT-PCR) and real time polymerase chain reaction (qPCR). Overall, these existing techniques have one or several disadvantages such as slow measurement, laborious, meticulous specimen preparation, and lack of sensitivity detection in non-laboratory settings typical of the point care (Guzmán & Kourí, 2004; Fang et al., 2009; Fletcher et al., 2010; Hue et al., 2011; Jin et al., 2016; Rahman et al., 2014; Rai et al., 2012; Saberi et al., 2016; Xu, 2012). To address these downsides, researchers are exploring surface plasmon resonance (SPR) sensor that is low-cost, simple to use, environment-friendly, high accuracy, and fast measurement (Parisi et al., 2008; Prabowo, 2018; Zhang et al., 2018b). However, the excellent and highly sensitive of SPR sensors are lacking to date. Therefore, in this work, we developed a quantitative SPR sensor with a novel biomolecular recognition to improve the sensitivity of SPR sensor. Anchoring a stable biorecognition element on the gold surface is important to support the antigen binding activity, allowing a significant change in angle of the reflectivity minimum, thus, particular suit for dengue virus detection.

Though many surface functionalization based-SPR sensors have been developed for optical sensing of DENV detection, their report on the sensing performance is very limited. Moreover, there are few works reported on dengue binding behaviour using structural measurements (Cui et al., 2012; Jahanshahi et al., 2014; Kamil et al., 2018; Khan et al., 2016; Loureiro et al., 2017; Singh et al., 2017; Valenga et al., 2012). Therefore, it is of interest to study the sensing performance and structural properties of the sensor film before and after detection of DENV E-proteins. In this work, several functionalized materials based-SPR sensor are promoted as plasmonic materials to enhance DENV detection. Owing to that, the detection performances of such sensitivity, binding affinity, and quantitation limits are compared to meet the most sensitive detection of DENV E-proteins.

In view of these factors, a rapid and quantitative method of using SPR optical sensor is developed to provide early detection of dengue virus.

1.6 Research objectives

The main objectives of this study are summarized as follows:

1. Develop the Au/Fe-NCC-CTAB/IgM, Au/CdSQDs-RGO/IgM, Au/CdSQDs-PAMAM/IgM, and Au/DSU/RGO-PAMAM/IgM thin films combined with SPR technique for detection of DENV E-proteins.

2. Analyse the sensing performances and structural properties of the thin films towards detection of DENV E-proteins.

3. Choose the optimum sensing performance towards DENV E-proteins detection.

4. Revise the Au/DSU/RGO-PAMAM/IgM thin film under optimized parameters for improved detection of DENV E-proteins.

1.7 Thesis organization

Chapter 1 provides a general introduction on dengue disease, envelope proteins of DENV, surface plasmon resonance phenomenon, self-assembled monolayers, problem statement, and objectives of the study. Chapter 2 presents a review on diagnosis methods for DENV, SPR biosensor for DENV detection, and development of self-assembled monolayer. Chapter 3 focuses on the fundamental of SPR in the aspect of polarization of light waves, surface plasmon waves, total internal reflection, excitation of surface plasmon waves, non-absorbing and absorbing dielectric medium, reflectance of multilayer structure, and SPR performances. Chapter 4 describes the experimental procedure of the developed sensor films used in this study. Chapter 5 shows the results and discussion of the Au thin film, Fe-NCC-CTAB thin film, CdSQDs-RGO thin film, CdSQDs-PAMAM thin film, and DSU/RGO-PAMAM thin film-based SPR optical sensor. Chapter 6 concludes the research findings made during the study and summarizes the performances for all developed sensing layers. The suggestions for future work are also given in this chapter.

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