



Faculty of Resource Science and Technology

**Chitosan Derived Nanoparticles –based Sensing Probes for Detection of
Japanese Encephalitis (JE) Antigen**

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Chitosan Derived Nanoparticles – based Sensing Probes for Detection of Japanese Encephalitis (JE) Antigen

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Japanese encephalitis virus (JEV), a mosquito-transmitted flavivirus that will lead to neurological diseases and death in humans in extreme cases. Up to date, there are currently no specific anti-viral medications and curative treatment for JEV. Therefore, early, effective and accurate diagnosis of JEV infection by using a point-of-care, simple, rapid and economical biosensor is very critical for the prevention and control of JEV outbreaks. A biosensor is an analytical device containing biocatalyst and a transducer for point-of-care screening which converts any diseases or harmful biological events to detectable signals. Designing and fabricating biosensors with a higher rate of reaction and sensitivity has to become popular among researchers due to the emergence of nanotechnology. In recent decades, carbon nanoparticles (CNPs) have attracted numerous researchers to study their nanoscience and nanotechnology potential comparing to other nanoparticles due to their special characteristics especially their optical and electrochemical properties which turn them into potential candidates for bioimaging, biosensing, drug delivery, and photodynamic applications and most importantly CNPs are easier to synthesize, environmental friendly and low cost. In this study, based on the electrical conductivity performance of CNPs derived from the various types of precursor characterized by using cyclic voltammetry, (CV) and electrochemical impedance spectroscopy, (EIS), chitosan CNPs with the smallest size and performed highest electrical conductivity are selected for further modification in order to fabricate a higher rate of reaction and sensitivity of electrochemical biosensor. Carbon nanoparticles modified on screen-printed carbon electrode (CNPs-SPCE) electrochemical biosensor strip has been successfully fabricated by immobilized JEV antibody onto the surfaces of carbon nanoparticles through amide bonds formed between amino groups of CNPs and carboxylic groups of JEV antibody. The analytical performance of the CNPs-

SPCE electrochemical biosensor strip was characterized using CV and EIS. CNPs-SPCE electrochemical biosensor strip exhibited a linear detection range of $1 - 20 \text{ ngmL}^{-1}$ with a low limit of detection (LOD) of 0.36 ngmL^{-1} (at $S/N = 3$) for JEV, detection sensitivity was 0.024 ngmL^{-1} for JEV and analysis results were obtainable within 10 minutes. In order to enhance the sensitivity and selectivity of the CNPs-SPCE electrochemical biosensor strip, the addition of Gold nanoparticles (AuNPs) onto CNPs to produce the hybrid Gold-Carbon nanoparticles (Au-CNPs) which provides a higher effective surface area, catalytic activity and electrical conductivity has been successfully fabricated. The analytical performance of hybrid Gold-Carbon nanoparticles modified on screen-printed carbon electrode (Au-CNPs-SPCE) electrochemical biosensor strips was characterized using CV and EIS. Au-CNPs-SPCE electrochemical biosensor strip exhibited a linear detection range of $1 - 20 \text{ ngmL}^{-1}$ with an extremely low LOD of 0.29 ngmL^{-1} (at $S/N = 3$) for JEV, detection sensitivity was 0.04 ngmL^{-1} for JEV and responding time is 10 minutes. The potential clinical application of this Au-CNPs-SPCE electrochemical biosensor strip was demonstrated by the detection of JEV in human serum while the selectivity of this biosensor strip was also proven by using Dengue antigen. In conclusion, virus infections can cause serious diseases to humans and animals as certain viruses could spread rapidly within a very short period. Hence, the fabrication of this accurate and fast biosensor for early detection of viruses is often crucial for clinical diagnosis and therapy.

Keywords: Carbon nanoparticles (CNPs), Japanese encephalitis virus (JEV), electrochemical biosensors, hybrid gold-carbon nanoparticles (Au-CNPs), chitosan

Alat Pengesan Sensor Berasaskan Nano-Zarah Karbon Diperolehi dari Kitosan untuk Mengesan Antigen Japanese Encephalitis (JE)

ABSTRAK

Virus Japanese encephalitis adalah sejenis flavivirus yang berjangkit melalui gigitan nyamuk boleh membawa kepada penyakit neurologi dan juga kematian jikalau jangkitan itu menjadi semakin teruk. Sehingga kini, tiada ubat anti-virus dan rawatan khas untuk JEV. Oleh itu, diagnosis yang lebih awal, cepat dan tepat bagi jangkitan JEV adalah sangat penting untuk mencegah dan mengawal wabak JEV merebak. Biosensor adalah peranti analisis yang mengandungi bio-mangkin dan transduser yang boleh mengesan apa-apa penyakit atau aktiviti biologi yang berbahaya dan mengubah kepada isyarat yang dapat dikenalpasti. Reka bentuk dan fabrikasi biosensor dengan kadar tindak balas dan sensitiviti yang tinggi adalah sangat popular di kalangan para penyelidik sejak kemunculan bidang nanoteknologi. Kebelakangan ini, CNPs telah menarik perhatian ramai penyelidik untuk mengkaji potensi dan keberkesanan kegunaannya dalam bidang nano-sains dan nanoteknologi berbanding dengan nano-zarah yang lain. Ini adalah kerana ciri khusus mereka terutamanya sifat optik dan sifat elektrokimia yang menjadikan CNPs sebagai calon pilihan utama untuk bioimaging, biosensing dan yang paling penting adalah CNPs mesra alam, proses penyediaan yang lebih mudah dan murah. Dalam kajian ini, berdasarkan kepada prestasi kekonduksian elektrik untuk pelbagai jenis CNPs yang diuji dengan menggunakan CV dan EIS, kitosan CNPs dengan saiz terkecil dan kekonduksian elektrik tertinggi dipilih untuk kajian selanjutnya supaya untuk menghasilkan biosensor yang mempunyai kadar tindak balas dan sensitiviti yang tinggi. Fabrikasi biosensor elektrokimia CNPs-SPCE berjaya disediakan dengan pembentukan ikatan amida antara kumpulan berfungsi $-NH_2$ dari CNPs dan kumpulan berfungsi $COOH$ dari antibodi JEV. Prestasi biosensor

elektrokimia CNPs-SPCE diuji melalui CV dan EIS. Strip biosensor elektrokimia CNPs-SPCE mempamerkan julat pengesanan linear antara 1 hingga 20 ngmL⁻¹ dan mempunyai LOD yang rendah iaitu 0.36 ngmL⁻¹ (pada S / N = 3) , kepekaan pengesanan untuk JEV ialah 0.024 ngmL⁻¹ dan keputusan analisis boleh didapati dalam masa 10 minit. Untuk meningkatkan kepekaan dan kepelbagaian biosensor elektrokimia CNPs-SPCE, penambahan AuNPs keatas CNPs untuk menghasilkan hibrid Emas-Karbon nano-zarzh (Au-CNPs) yang mempunyai jumlah luas permukaan yang lebih tinggi, aktiviti mangkin, kekonduksian elektrik dan bio-keserasian yang jauh lebih berkesan. Prestasi biosensor elektrokimia Au-CNPs-SPCE mempamerkan julat pengesanan linear 1 hingga 20 ngmL⁻¹ dengan LOD yang sangat rendah iaitu 0.29 ngmL⁻¹ (pada S / N = 3) kepekaan pengesanan ialah 0.04 ngmL⁻¹ untuk JEV dan masa yang bertindak balas adalah 10 minit. Potensi penggunaan klinikal untuk biosensor elektrokimia Au-CNPs-SPCE ini ditunjukkan oleh pengesanan JEV dalam serum manusia dan kajian pemilihan virus turut dibuktikan dengan menggunakan antigen denggi. Kesimpulannya, jangkitan virus boleh membawa penyakit yang serius kepada manusia dan haiwan disebabkan terdapat sebahagian virus tersebut boleh menyebar dengan cepat dalam tempoh yang sangat singkat. Oleh itu, pengesanan jangkitan virus dengan lebih awal, berkesan dan cepat adalah sangat penting untuk diagnosis dan rawatan di klinik.

Kata kunci: Nano-zarah karbon (CNPs), virus Japanese encephalitis (JEV), biosensor elektrokimia, hibrid emas-karbon nano-zarah (Au-CNPs), kitosan

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LIST OF ABBREVIATIONS

Au-CNPs	Hybrid Gold-Carbon nanoparticles
Au10-CNPs	Hybrid 10 nm Gold-Carbon nanoparticles
Au20-CNPs	Hybrid 20 nm Gold-Carbon nanoparticles
Au40-CNPs	Hybrid 40nm Gold-Carbon nanoparticles
Au-CNPs-SPCE	Hybrid Gold-Carbon nanoparticles modified on screen-printed carbon electrode
BSA	Bovine serum albumin
CNs	Carbon nanomaterials
CNTs	Carbon nanotubes
CNPs	Carbon nanoparticles
CNPs-SPCE	Carbon nanoparticles modified on screen-printed carbon electrode
CV	Cyclic voltammetry
DNA	Deoxyribonucleic acid
EDC	N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EIS	Electrochemical Impedance Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
GCE	Glassy carbon electrode
GO	Graphene oxide
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
LOD	Limit of detection
MAC-DOT	IgM capture dot enzyme immunoassay

MAC-ELISA	IgM antibody capture enzyme-linked immunosorbent assay
MW-CNT	Multiple wall carbon nanotube
NDs	Nanodiamonds
NHS	N-Hydroxysuccinimide
PBS	Phosphate buffer saline
PRNT	Plaque reduction neutralization test
R_{et}	Electron-transfer resistance
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SAM	Self-assembly method
SCE	Standard calomel electrode
SEM	Scanning electron microscopy
SHE	Standard hydrogen electrode
SPCE	Screen-printed carbon electrode
SW-CNT	Single wall carbon nanotube
UNIMAS	Universiti Malaysia Sarawak
UV-Vis	Ultraviolet visible Spectroscopy
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Background and Problem Statement

Japanese encephalitis virus (JEV), a mosquito-transmitted flavivirus consisting of a positive-sense single-stranded ribonucleic acid (RNA) genome which acts as messenger RNA encoding a single open reading frame (Yun & Lee, 2014). This will then lead to neurological diseases and death in humans in extreme cases (Li et al., 2015; Bharucha et al., 2018). World Health Organisation (WHO) has reported that JEV outbreaks first happened in Japan during the 19th century. In recent years, JEV has become a major public health concern globally as there are estimated 70,000 human cases and 20,000 deaths annually (Nain et al., 2016). Mansfield et al. (2017) pointed out some reproductive problems such as abortion, still-birth and birth defects happening to adults infected by JEV. On the other hand, JE is the major cause of encephalitis and 75% happened to children less than 15 years old in Asia (Mansfield et al., 2017). According to Zanin et al. (2003), vaccination for JEV has been available and used internationally since the 1930s and the commercially used vaccines now in the market are JE-VAX and JE-VC, respectively. However, Mansfield et al. (2017) reported that hypersensitivity and licensed reason in certain countries are some of the reasons for the uncontrollable outbreaks of JEV in endemic countries. Accurate diagnose is therefore important to prevent and control the JEV outbreaks.

Some conventional diagnostic devices have been used for the diagnosis of JEV infection, for example, enzyme-linked immunosorbent assay (ELISAs) method, plaque reduction neutralization test, reverse transcription-polymerase chain reaction (RT-PCR) and virus isolation (Hobson-Peters, 2012). These methods involve costly equipment and

specialized expertise to run the laborious and slow analyses. A simple nitrocellulose membrane-based immunoglobulin M (IgM) capture dot enzyme immunoassay (MAC DOT) was developed and commercialized for the diagnosis of JE (Solomon et al., 1998). The IgM antibody capture (MAC ELISA) is the first-line serological assay recommended by the World Health Organization (WHO) for JE diagnosis. There are several types of MAC ELISA kits currently available commercially. However, studies conducted on three of the most common MAC ELISA kits (Panbio JE-Dengue IgM combo ELISA, XCyton JEV CheX and InBios JE Detect) have shown that they have low sensitivities which ranged between 17% and 57% (Robinson et al., 2010). Furthermore, all these diagnostic methods are confined to qualitative analysis only.

Majority of the JE cases happened in rural areas in Southern and Eastern Asian Countries, where access to diagnostic laboratory facilities is very limited. Mansfield et al. (2017) mentioned JEV infection is difficult to be detected due to a short duration of viremia. Hence, a portable biosensor system that can provide point-of-care, rapid, sensitive and economical diagnostic tool is highly needed for such settings. Undeniably, it has drawn the attention of many researchers in the related field to explore and design different configurations and approaches to preparing biosensors. This is important as many dangerous bacterial infections could be triggered by as low as 10 organisms. Various configurations of biosensors are being explored recently which includes antibody-based system, enzyme-based detection, and DNA-based sensors. The biosensor has proven its high potential in terms of ability to shorten the detection time from 2 to 4 days to less than an hour *via* a simple target extraction method (Wang & Dai, 2015). This is an important discovery in the medicinal field which shows integration in ensuring a fast and easy way to obtain a signal from biological events (Liu & Guo, 2012).

Generally, a biosensor is one of the emerging technologies showing high potential in obtaining rapid results and the device that converts the biological response to an electrical signal using a transducer (Malhotra et al., 2017). Biosensors are divided into different types such as electrochemical sensors, amperometric biosensors, blood glucose biosensors, potentiometric biosensors, piezoelectric biosensors, and electrochemical biosensor. Biosensors have a greater potential in terms of sensitivity and selectivity when compared to conventional biosensors. Consequently, biosensors are widely used in industrial processing and monitoring, environmental pollution control, agriculture, food industries and most importantly in the field of medicinal diagnostics. For instance, reusable sensors permitted calibration and quality control to carry an important role in improving the efficiency of patient care as well as replacing the present test which is slow and is relies a lot on manpower (Malhotra, 2017). This is important as a virus could spread rapidly and accurate detection in a short time is important to treat the patients.

Figure 1.1 shows the working principle of the electrochemical sensor. An electrochemical biosensor is based on the reaction of enzymatic catalysis and containing a substrate which includes three electrodes namely counter, reference, and working type (Mahorta et al., 2017). The biological material is immobilized *via* the covalent binding and then is allowed to be in contact with the transducer. Analyte will later bind to the biological material which in turn produces the electronic response as shown on the biosensors.

Ravi (2019) described the electrical signal from the transducer is normally low and superimposed when there are high-frequency signals overlapping due to certain electrical interference baseline. Hence, a 'reference' baseline will derive from a similar transducer without any biocatalyst membrane then continue by amplifying a resultant signal, electronically filtering the unwanted signal noise. The analogue signal produced is converted

to a digital signal followed by passing to a microprocessor stage where the data is processed, manipulated to desired units and output to a display device.

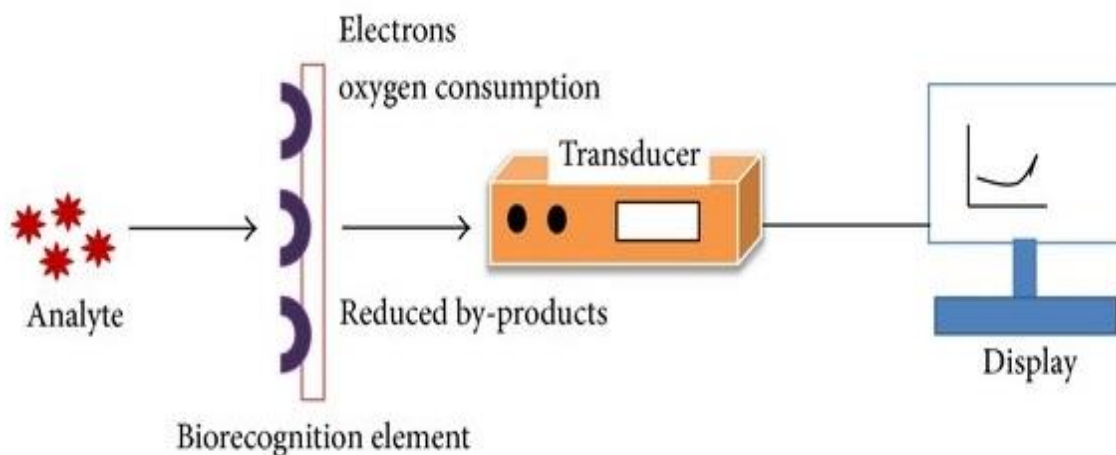


Figure 1.1: Schematic diagram of the working principle of the electrochemical biosensor (Dhull et al., 2013).

To date, electrochemical biosensors also gained numerous interests as a reliable tool for the diagnosis of infectious diseases as the turbidity or absorbance of the sample would not disturb their accuracy (Bakker & Telting-Diaz, 2002). Moreover, they are extremely sensitive, produce readout fast and easily miniaturized and do not require complicated instruments (Pejcic & Marco, 2006). Thus far, not that many electrochemical biosensors for JE diagnosis have been reported. Recently, an electrochemical biosensor for rapid detection of JE antigen by immobilized serum antibody on a silanized surface of an interdigitated sensor has been fabricated. Stable electrochemical signals were obtained from the JE antibody/antigen binding reaction in as short as 20 minutes. A detection limit of only 0.75 micrograms per millilitre was reported, but the performance of the biosensor is still not good enough for clinical diagnosis (Huy et al., 2011).

To overcome the sensitivity and response time problems, many research efforts have been devoted to enhance the detection sensitivity and response time of biosensors by the

incorporation of nanomaterials into electrodes of biosensors. In general, materials with a one-dimension nanoscale range (<100 nm) were known as nanostructured materials. Nanostructured materials were categorized into three classes namely zero-dimensional nanostructures (nanoparticles); one-dimensional nanostructures (nanowires, nanofibers, and nanotubes); and two-dimensional nanostructures (thin films) (Cao et al., 2008). Recently, studies and fabrications of nanostructured materials such as nanoparticles, nanoforest, nanotube and nanowires had attracted considerable scientific and technological interests due to their chemical, catalytic, mechanical, electrical, optical, and magnetic properties which could be varied depending on their particle sizes and morphological structures (Theerthagiri et al., 2019). The high surface to volume ratio of nanoparticles will lead to a substantial increase in the loading of recognition elements on the electrodes and more efficient electrons transfer and resulted in the increase of the sensitivity of the biosensors (Merkoci, 2010).

Carbon, as one of the most abundant elements on earth, has been attracting a great attention of researchers due to its ability to form different types of hybridization states (sp , sp^2 , and sp^3) that would form a wide range of allotropes such as diamond, the hardest material and graphite, one of the softest substances. Unser et al. (2015) mentioned carbon nanostructures have been a subject of intensive research due to the emerging of nanotechnology and the discovery of fullerenes in 1985. Since then, carbon-based nanomaterials such as carbon nanotubes (CNTs), CNPs and graphene have been synthesized and broadly investigated due to their superior electronic, physical, optical and magnetic properties. Carbon-based nanostructured materials such as CNTs and graphene which can be prepared easily from low-cost precursor without polluting the environment have been extensively studied for the fabrication of electrochemical biosensors applications due to their excellent electrochemical properties, ultra-lightweight, and excellent chemical stability

(Shao et al., 2010; Moharana et al., 2019). However, the synthesis methods for CNTs and graphene are complex, whereas commercially available CNTs and graphene are very costly.

Among all the carbon-based nanostructured materials, CNPs is chosen as a substrate for immobilisation for biosensor because CNPs is one of the carbon-based nanostructured materials which have attracted high attention in biosensing due to their mechanical, electrical, thermal, optical and structural properties (Rezaei et al., 2016). The main reason that gives rise to an ample mechanical strength and high electrical and thermal conductivity of CNPs is the sp^2 hybridisation of carbon atoms in the structure as it could form a stronger bond between the carbon atoms than those of sp and sp^3 hybridisations. The presence of the CNPs layer on the surface of Screen-printed carbon electrode (SPCE) could contribute significantly to the promotion of electrochemical activity of the sensing electrode which would, in turn, enhance the electronic signals (Wang et al., 2018).

As reported by Tang et al. (2010), CNPs which was highly conductive and nontoxic, provided a high capacity nanoparticulate immobilization surface and a facile pathway for electrons transfer. In addition, it exhibited a high bio-electrocatalytic response towards enzyme-substrate and hence could obtain a wide dynamic range from 0.02 to 4.0 ng/mL with a low detection limit of 10 pg/mL toward AFP. The common method to increase the sensitivity of a sensor is by increasing the surface contact area *via* nanostructure modification as in controlling the size and morphology of nanostructure used. These methods had been applied on different types of biosensors which includes glucose biosensor (Zheng et al., 2018), protein sensing (Suthanthiraraj & Sen, 2019), gas sensing (Yeh et al., 2017), DNA sensing (Hajihosseini et al., 2016; Chen et al., 2018), and multivalent interactions (Tung et al., 2019).

In recent years, noble metal nanoparticles such as gold, silver, copper, and aluminium have drawn the attention of researchers in preparing biosensor for detection of various diseases due to their obvious sensitivity towards biological binding (Unser et al., 2015). A significant increase in the limit of detection obtained even though only a small amount of materials being used. Gold nanoparticles (AuNPs) labelled biosensor had been developed by Wang and Dai (2015) in detecting the presence of *E.coli* 1257:H7 and the detection was completed within 45 minutes with a lower limit of detection at 10_1 cfu/ml at a dynamic range of 10_1 to 10_6 cfu/ml. However, researchers faced various challenges such as detection of membrane-associated species, the limit of detection and selectivity when using these noble metal nanoparticles.

Metal nanoparticles such as gold and silver nanoparticles have been widely studied for the fabrication of biosensors due to their convenient labelling with biomolecules (Siangproh et al., 2011). Meanwhile, metal nanoparticles exhibited various interesting properties in terms of optical, electronic and magnetic properties. This had become one of the main reasons for metal nanoparticles being used in enhancing biosensor sensitivity. The signals could be amplified by increasing the capture efficiency of targeted molecules through their optical and electronic properties of metal nanoparticles. Wang and Dai (2015) discussed the utilization of (AuNPs) and polymeric nanoparticles in signal amplification.

Localized surface plasmon resonance (LSPR) is another leading technique recently as it is a label-free biosensor, with both facile, colorimetric detection and the ability interfacing with portable and multiplex devices (Unser et al., 2015). The potential of LSPR is high and shows a great impact in the related field, but still, there are few challenges and limitations. The main challenge lies in increasing the sensitivity of devices to improve the limit of detection of targeted analytes. Apart from that, a sensitive electrochemical DNA

biosensor with a 20 pM limit of detection and well-responded in the stimulated real samples detection was developed by Hajihosseini et al. (2016). According to the group, a single-stranded DNA probe was immobilized on a graphene oxide/gold nanoparticle modified glassy carbon electrode (GO/AuNPs/GCE) for a hybridization reaction. Pan et al. (2015) prepared graphene-gold nanoparticle composite materials biosensor. A detection limit as low as 1 nM was achieved which demonstrated that the biosensor is a promising and reliable tool for rapid detection.

In addition, for further enhancement of biosensors studies, Li et al. (2017) modified SPCE by using AuNPs. AuNPs modified SPCE was prepared *via* combining vortex-assisted ionic liquid dispersive liquid-liquid microextraction (IL-DLLME) with microvolume back extraction which successfully determined mercury in water samples and non-digested urine samples with LOD lower than 0.2 µg/L and 0.5–1.5 µg/L, respectively while Lv et al. (2019) fabricated gold nano-ellipsoid array by immobilized antigens on the surface of nano-ellipsoid and then was further integrated into the microfluidic chip biosensor and successfully achieved a detection limit of 1 ng/mL and Xu et al. (2017) discovered a sensitive magnetic porous pseudo-carbon paste electrode (MPPCPE) electrochemical sensor showing that the fabricated MPPCPE produced a detection limit as low as 0.005 nM. The achievement of this great potential electrochemical sensor as point-of-care devices encouraged the further exploration of researchers in this field.

There have been researchers actively studied on the development of biosensors for the detection of veterinary viral pathogens such as flaviviruses from JEV, influenza, pestiviruses, foot-and-mouth disease virus and more. However, none of these biosensors are commercially available yet. Hence, it can be concluded that biosensing for veterinary

pathogens is still in its initial steps and need more work to instil the confidence level of global technologies to push the development for JEV detection (Ayyar & Arora, 2013).

In this study, CNPs were synthesized by crosslinking chitosan with tripolyphosphate *via* the ionic gelation technique (Fan et al., 2012). These CNPs synthesized are further tailored in terms of physical, chemical, and electrochemical properties to optimize the sensing of the JE antigen. Subsequently, CNPs synthesized were functionalized with the JE antibody. The drop-coating technique of CNPs onto the SPCE was used to develop an extremely sensitive, fast, and reliable detection carbon nanoparticles modified on screen-printed carbon electrode (CNPs-SPCE) electrochemical biosensor strip. A hybrid gold-carbon nanoparticle modified screen-printed carbon electrode (Au-CNPs-SPCE) electrochemical biosensor strip was prepared in the later studies in detecting JEV. The utilization of different sizes of synthesized nanoparticles in electrochemical properties in detecting JEV was investigated.

1.2 Objectives

The objectives of this research include:

- i. To synthesize and characterize CNPs functionalized with Japanese encephalitis (JE) antibody.
- ii. To optimize the physical, chemical and electrochemical properties of CNPs for optimum sensing of JE antigen.
- iii. To develop a highly sensitive CNPs based electrochemical biosensor for fast and reliable detection of JE antigen.

1.3 Chapter Summary

Generally, this research focuses on the synthesis and optimisation of more sensitive lower value of LOD, point-of-care, rapid, economical, portable and reliable of chitosan

derived carbon nanoparticles-based sensing probes for detection of JEV which is very crucial for those rural area that is lacked proper medical facilities.

In Chapter 1, it is clearly stated the problem that facing globally as well as Southeast Asian for development of the point-of-care, simple, easy to operate, economical, rapid and sensitive devices or methods for detection of JEV and for the purpose of early and accurate diagnosis which is critical for prevention, control and management of JEV infection while also describe the background and justification of this study. Chapter 2 summarizes the literature review on different types of biosensors, different types of carbon-based nanostructures, synthesis of CNPs, electrochemical characterization of biosensors followed by the immobilization procedure on the electrode surface, and an overview for JEV and method of diagnosis.

In Chapter 3, different types of carbon-based nanoparticles or precursors for further immobilisation of electrochemical biosensor (chitosan, starch, fibrous cellulose, carboxymethylcellulose, ascorbic acid and citric acid) are synthesized and characterized. The carbon-based nanoparticles with smallest size, exhibit highest magnitude of current in Cyclic voltammetry (CV) test and smallest value of electron-transfer resistance (R_{et}) from Electrochemical Impedance Spectroscopy (EIS) test will be selected as a substrate for further immobilization with JEV antibody in electrochemical immunosensor for the detection of JEV which will discuss in Chapter 4 and for fabrication of hybrid gold-carbon nanoparticles (Au-CNPs-SPCE) that will report in Chapter 5.

In Chapter 4 reports on the CNPs-SPCE based electrochemical biosensor strip for the detection of JEV. Among all the carbon-based nanoparticles synthesis in the previous chapter, the smallest size of chitosan CNPs with highest magnitude of current in CV test and

smallest value of electron-transfer resistance (R_{et}) from EIS test is chosen to proceed for functionalised with JEV antibody. The successive functionalisation of JEV antibody immobilized CNPs is then immobilized onto SPCE electrode for JEV detection. As it had been reported that metal nanoparticles such as gold nanoparticles have been widely studied for the fabrication of biosensors due to their convenient conjugate with biomolecules (Siangproh et al., 2011) and also exhibited various interesting properties like optical, electronic and magnetic properties. Therefore, the CNPs-SPCE based electrochemical biosensor synthesis from Chapter 4 and different size of AuNPs (10nm, 20nm and 40nm) are used to fabricate a hybrid gold-carbon nanoparticle (Au-CNPs-SPCE) electrochemical biosensor strip for JEV detection is carried out in Chapter 5.

The final chapter (Chapter 6) presents the concluding remarks from these studies with recommendations for future research works.

CHAPTER 2

LITERATURE REVIEW

2.1 Biosensor

A biosensor is an analytical device containing biocatalyst and a transducer for point-of-care screening which converts any diseases or harmful biological events to detectable signals (Liu & Guo, 2012; Baca et al., 2015). A biosensor contains three main electrodes namely a working electrode, a counter electrode and a reference electrode. Designing and fabricating biosensors with a high rate of reaction and sensitivity has become popular among researchers due to the emergence of nanotechnology (Rezaei et al., 2016). Various types of nanostructures such as carbon nanotubes, nanowires, nanoparticles, nanopores, nanoclusters, nanofibers, and graphene were employed in electrochemical biosensors. Arduini et al. (2016) described a nanomaterial-based electrochemical biosensor that shows significant advantages over conventional biosensors which includes effective sensing mechanism, high spatial resolution for localized detection, facile integration with standard wafer-scale semiconductor processing and label-free, real-time detection in a non-destructive manner. Biocatalytic with examples and affinity sensors with examples will be discussed in Section 2.1.1 and Section 2.1.2 respectively.

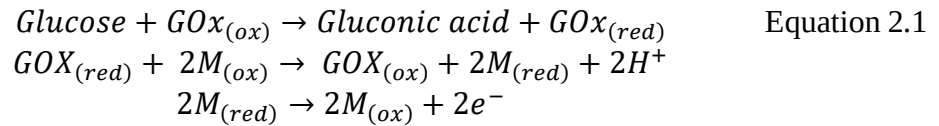
2.1.1 Biocatalytic Sensor

Baca et al. (2015) explained biocatalytic sensor is a device used to detect a target substrate with the help of an immobilized enzyme. Biocatalyst recognizes the respective analyte and hence generates a complex as a product that is stable as a transition state which will then detect the change of analyte or product *via* biosensor. Examples of biocatalytic

sensors include glucose sensor, xanthine sensor, and lactate sensor which utilizes glucose oxidase, xanthine oxidase, and lactate oxidase respectively (Christwardana & Frattini, 2018).

2.1.1.1 Glucose Sensors

Glucose sensors are used widely in measuring blood glucose concentration of a diabetes patient by pricking the finger to draw blood, which is then applied to a disposable “test-strip” (Zhang & Hoshino, 2014). An electrode in the glucose sensor plays an important role in measuring the current generated by reducing properties of glucose when an enzymatic reaction occurs (Anusha et al., 2014). Christwardana and Frattini (2018) emphasized the use of glucose oxidase (GOx or GOD) becoming the gold standard for glucose sensing. The working principle of glucose biosensor was first mentioned by Clark and Lyons in the year 1962 where a thin layer of GOx was entrapped by a semipermeable membrane and then measurement of the oxygen consumed by the enzyme-catalyzed reaction was detected according to the Equation 2.1 as shown below:



According to Christwardana and Frattini (2018) glucose reacted with the enzyme $\text{GOx}_{(ox)}$ followed by reduction of enzyme $\text{GOx}_{(red)}$ which then reduced two mediator $\text{M}_{(ox)}$ ions to $\text{M}_{(red)}$. Reduced mediators formed were later re-oxidized at the electrode and hence providing an electrical signal to be measured (Ferri et al., 2011; Zhang & Hoshino, 2014).

2.1.1.2 Xanthine Sensors

Xanthine is a precursor of uric acid resulting from purine degradation metabolism in the human body (Muragaiyan et al., 2014). Gout, xanthinuria, hyperuricemia, renal failure, and toxemia during pregnancy are some of the abnormalities of metabolites concentrations

(Nguyen et al., 2019). In the past, the method of analyzing xanthine such as capillary column gas chromatography, chemiluminescence, spectrophotometry, and high-pressure liquid chromatographic (HPLC) requires skilled operators and time consuming (Zhang et al., 2019). Consequently, the electrochemical method which is simple, high sensitivity, and rapid response attract attention in research. Ojani et al. (2013) fabricated a high sensitivity electrochemical sensor for xanthine based on poly(L-methionine) modified glassy carbon electrode which achieved a detection limit of 0.004 μM . A MIL-101(Cr) with porous cages and a large specific surface was selected by Zhang et al. (2019) as the host framework for loading Pt nanoparticles to construct a non-enzymatic electrochemical platform for sensing xanthine at a low detection limit of 0.42 μM .

2.1.1.3 Lactate Sensors

Lactate is a common analyte in food processing industries as it presents in the fermentation of cheese, yogurt, butter, pickles, and so on. Monitoring the concentration of lactate is important in ensuring the freshness of the food product (Malhotra et al., 2017). Lactate is formed from the anaerobic metabolism of glucose in muscle and the lactate sensor plays an important role in monitoring health and exercise in humans (Kai et al., 2018). Kai et al. (2018) also explained the importance of monitoring lactate because as cellular proton buffering capacity in body exceeded, cellular pH decreases and hence causing cell acidosis which in turn disrupts the performance of muscles. Yang et al. (2016) developed a lactate-oxidase-based flexible lactate sensor that could last four weeks with an average surface area normalized sensitivity of 950 nA ($\text{cm}^{-2}\text{mM}^{-1}$). As reported by them, gold and IrO_x electrode films act as the primary materials due to the rough surface of IrO_x provides an improvement insensitivity.

2.1.2 Bio-affinity Sensors

Bio-affinity sensors relies on the selective binding target analyte to a surface-restricted ligand partner such as DNA, cell, oligonucleotide, and antibody. As a result, bio affinity sensors with a pre-formed metastable ligand-receptor complex have been used widely in the determination of thyroxine (T4), biotin and insulin (Liu et al., 2018). Examples of bio-affinity sensors include immunoassay and immunosensors, and DNA hybridization biosensors and discussed in Section 2.1.2.1 and Section 2.1.2.2 respectively.

2.1.2.1 Immunoassays and Immunosensors

Immunosensors work by combining molecular recognition properties of an antibody with the high sensitivity of an enzyme-based analytical method (Liu et al., 2018). There are two types of immunosensors namely non-labelled immunosensor and labelled immunosensor where the selectivity depends on the immunochemical affinity of antigen for its corresponding antibody and chemical amplification of a labelling enzyme for sensitivity respectively. Steps of using immunosensor for immunoglobulins (IgG) with an amperometric oxygen electrode has been reported by Murugaiyan et al. (2014) as follows:

Step 1: Oxygen electrode with a membrane is bound onto the anti-IgG antibody.

Step 2: Label-free IgG with enzyme catalase.

Step 3: Mix a known amount of labelled IgG with a sample with an unknown amount of unlabelled IgG.

Step 4: Place the mixture into the chamber of oxygen electrode while labelled and unlabelled IgG will compete for antibody on the membrane.

Step 5: Rinse the sensor to remove all non-specifically associated IgG.

Step 6: Fill sensor with hydrogen peroxide.

From the test, the more unlabelled IgG that is present, the lower the amount of the labelled IgG and lowers the rate of oxygen evolution (Borgmann et al., 2011; Murugaiyan et al., 2014). Immunoassay and immunosensor methods show accurate results, but the time needed for antigen-antibody coupling took several hours (Liu et al., 2018).

2.1.2.2 DNA Hybridization Biosensors

Murugaiyan et al. (2014) reviewed DNA biosensor is based on nucleic acid recognition processes in the detection of DNA damage and interactions. The advantage of DNA biosensor over immunosensor is that nucleic acid recognition layers can be readily synthesized and regenerated for multiple uses (Hajihosseini et al., 2016). Lv et al. (2019) reported on the working principle of DNA hybridization biosensor. According to the group, peptide nucleic acid (PNA) provides a new vista in the biosensor as the sugar-phosphate in PNA is replaced with pseudo peptide which will then be extended onto transducer surfaces of biosensor followed by attributing sequence specificity onto DNA biosensors that could detect even a single base mismatch. A DNA sequence that could hybridize to the complementary DNA sequence are the DNA dendrimers and hence increased hybridization capacity with a substantially amplified response when immobilizing these dendritic nucleic acids onto the physical transducers imparts.

2.2 Carbon-Based Nanostructures

Nanostructured materials refer to materials with at least one dimension with a size below 100 nm (Rezaei et al., 2016; Liu et al., 2017). According to Asadian et al. (2019), nanostructures provide a promising framework for applying the ‘bottom-up’ approach introduced by Feynman (1959), and they have attracted numerous researchers to study their potential nanoscience and nanotechnology applications (Toumey, 2008). The ‘bottom-up’ approach is used in assembling atoms or molecules to build nanomaterials piece by piece in

a controlled manner. Carbon presents in different types of allotropes which are categorized into zero-dimensional (0D), one-dimensional (1D), two-dimensional (2D), and three-dimensional (3D) carbon nanostructures and are shown in Figure 2.1.

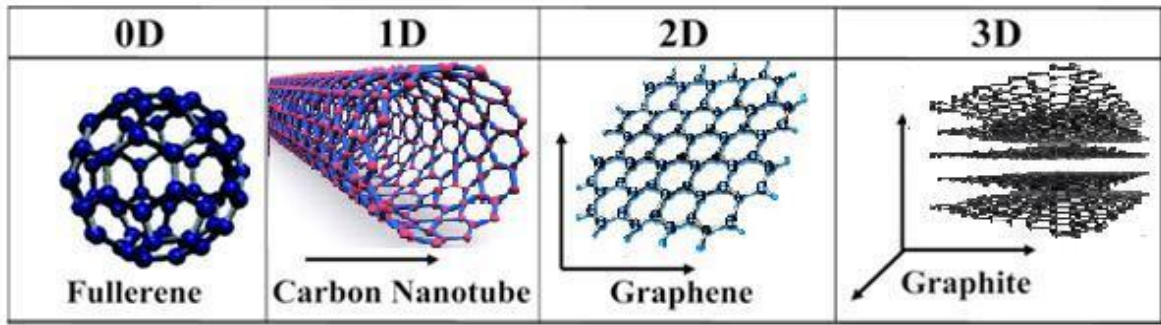


Figure 2.1: Carbon nanostructures with different dimensions (Mohamed, 2017).

Carbon nanostructures exhibit unique electrical, mechanical, physical, and chemical properties (Mendes et al., 2018). Furthermore, carbon nanostructures with high surface area enable it to be functionalized easily and cheap and simple production steps gain most attraction from various groups of researchers. Different types of carbon nanostructures will be further discussed in Section 2.2.1 to Section 2.2.6 which include fullerenes, carbon nanotube (CNT), graphene, nanodiamonds, nanofibers, and nanoparticles.

2.2.1 Fullerenes

Fullerene is one of the allotropes of carbon with the formula C_{60} with the shape of a hollow sphere, ellipsoid, and tube (Rao & Seshadri, 2019). It consists of 20 hexagonal and 12 pentagonal rings where each carbon atom is bonded to three others and is sp^2 hybridized with asymmetry closed cage structure (Zhao et al., 2018). The existence of π -electron in nature allows the conjugated π -electron systems of these hollow spheres to facilitate a rich chemical property. Despite that, fullerene led to tremendous research as the mediator between recognition site and electrode in biosensor due to the properties such as easy

chemical modification, conductivity, good biocompatibility, and electrochemical properties (Pilehvar & De Wael, 2015). Yanez et al. (2017) demonstrated an excellent working electrode using a partially reduced fullerene modified electrode. Although fullerene shows a promising advantage in biosensing devices by enhancing the rate of electron transfer, the main concern that draws the attention of researchers is controlling the condition as well as the performance of fullerene modified biosensor in detecting and measure various types of the analyte.

2.2.2 Carbon Nanotubes

A nanotube is a hollow tube made from a pure element. Carbon nanotubes (CNTs) have a typical diameter of 1-5 nm and could be up to several hundred microns long. They form a closed and relatively stable system that can allow ballistic electron (hole) to be transported along the axis of the carbon nanotube. CNTs have a single wall (SW-CNT) or multiple walls (MW-CNT). Based on their structural symmetry, CNTs can be metallic or semiconducting materials (O'connell, 2018; Zhao et al., 2018). Christwardana and Frattini (2018) reported the unique mechanical and electronic properties of CNT which includes low bulk densities, high Young's Modulus, high tensile strength, chemically and environmentally stable, high thermal conductivity, low bandgaps, able to adsorb and bond with proteins *via* chemical bond formation and physisorption, and last but not least their ability to get functional biocatalyst structures for biodevices. Asadian et al. (2019) reported that CNTs with a permanent capacity to mediate rapid electron-transfer, and ability to pull nearby molecule towards them helps to improve solubility and biocompatibility.

2.2.3 Graphene

Graphene acts as the basic building block for graphitic materials is a two-dimensional crystalline with a single layer of sp^2 hybridized carbon atoms arranged in a honeycomb

structure (Huang et al., 2012b; Hyun et al., 2013; Zhao et al., 2018). Inagaki et al. (2013) reviewed the methods of preparing graphene to include mechanical cleavage of crystal, exfoliation of graphite through intercalation compound, chemical vapor deposition on different substrate crystals, and organic synthesis process. As reported by Zhao et al. (2018), graphene has a maximum surface-to-volume ratio due to its simple bonding configuration in a 2D manner which further enhances the exposure of every carbon atom to the environment. This is rather important as a small change in the environment could lead to a drastic change in electrical properties and then detected by the biosensor. Meanwhile, graphene contains only carbon atom allowing enhancement in functionalizing selected immobilize analyte on their surfaces (Al-Jumaili et al., 2017). Although graphene shows promising advantages in biosensor, a large-scale production of high-quality graphene is still difficult and causes graphene to not attract much attention currently (Pan et al., 2015).

2.2.4 Nanodiamonds

Nanodiamonds (NDs) with special features including chemical inertness, biocompatibility, hardness, optical transparency, and high thermal conductivity makes most researchers throw attention to NDs in various applications like sensors, drug delivery, and bio labelling (Rao & Albaseer, 2018) are truncated semi octahedral carbon structures with the size approximately 2-8 nm with several physical properties such as charged surface facets, and surface containing chemical functional groups which enable it to deliver small molecules, proteins, and nucleic acids (Wang et al., 2018). Bacakova et al. (2016) mentioned NDs consisting of nitrogen-vacancy colour at centre plays an important role in nanoscale biosensing. Bacakova and co-workers demonstrated on immobilizing NDs onto a gold electrode *via* direct adsorption method in determining the concentration of glucose and lactate.

2.2.5 Nanofibers

Lin et al. (2018) reported that nanofibers can be considered as two separate words “nano” and “fibers” in which “nano” was used to describe anything on a scale of 10^9 of the reference units while the word “fibers” was defined as a slender, elongated, threadlike object or structure. Nanofibers consisting of vertically aligned one-dimensional (1D) materials have been used in electrochemical energy storage, which provides good mechanical and electrical properties of 1D nanomaterials to enhance the electrochemically active surface area and packing density (Lin et al., 2018). Rezaei et al. (2016) reported that nanofibers show the substantially good foundation for biosensor applications due to the high specific surface area, large pore volume per unit mass, and tailorable chemical surfaces. However, there were several challenges to be overcome to deposit vertically aligned nanoforest onto a large area of flexible conductive substrates.

2.2.6 Nanoparticles

Nanoparticles attracted growing attention in recent years due to their potential application in biomedical research because of 10^9 size which falls into the range of biological molecules and structures (Jazayeri et al., 2016). Various researchers had proven different characteristics of nanoparticles in terms of purity, size, shape, and surface charge took up the role in influencing therapeutic and diagnostic efficiency (Zhao et al., 2018). These characteristics supported a variety of applications containing drug and gene delivery, bioimaging, vaccine development, biosensors, and therapies (Jazayeri et al., 2016). The preparation of nanoparticles with desired quality, and low cost by using a simple method that can be scaled up to a larger scale is important.

2.2.6.1 Preparation of Carbon Nanoparticles

Synthesis method of CNPs are categorized into two different approaches, namely, “top-down” and “bottom-up” where top-down approach refers to breaking down larger carbon structures such as graphite, soot, carbon nanotubes or activated carbon into carbon nanoparticles by using harsh chemical or physical conditions, meanwhile, bottom-up methods involve synthesizing small carbon particles from carbonaceous molecular precursors such as citrate, carbohydrates and small alcohols (Asadian et al., 2019).

“Top-down” synthesis method such as arc-discharge had been reported by Asadian et al. (2019) using crude single-walled-carbon nanotube (SW-CNTs) soot as a precursor. SW-CNTs are oxidized with 3.3 M nitric acid followed by extraction using an alkaline solution of pH 8.4. Despite that, the laser ablation method where carbon target as a precursor is another popular method as UV pulsed laser irradiation of carbon targets immersed in water at room temperature for min (Shen et al., 2014).

However, the ‘bottom-up’ method has been chosen over the “top-down” method in preparing CNPs due to the simplicity of the “bottom-up” approach. Bayda et al. (2017) reported the method of preparing CNPs through carbonization of commercial tea followed by oxidation with nitric acid using a green source, “black tea”, as a precursor. Carbonized carbon is prepared by heating “black tea” at 200 °C for 3 hr, followed by evaporation of water before it is heated again for 5 h. Dried carbonized carbon was dispersed in 0.065 mol nitric acid and undergoes reflux at 80 °C for 20 h. Bayda and team claimed this synthesis is economically friendly as a carbon source is inexpensive and these non-toxic CNPs can be synthesized easily in large-scale production with tunable optical properties.

Schwartz et al. (2018) and co-worker prepared CNPs via hydrothermal synthesis with diameters ranging from 10 – 500 nm through variations of a sugar source, the concentration

of agave (as a sugar source) and incubation time. A revolver is set-up and could rotate continuously for 30 min followed by removing it from heat while adding 3 mL of deionized water. Lastly, sonicated the solution for 2 min and then syringe filtered the solution to obtain CNPs. On the other hand, Li et al. (2015) and co-workers also reported on a similar method in preparing CNPs where folic acid is used as a precursor. As reported by them, folic acid is first dissolved in the mixture of the solvent containing deionized water and hydrochloric acid before transferring to an autoclave and heated 260 °C for a period of time. The product formed is finally centrifuged for 15 min in order to remove the less-photoluminescent deposit.

2.2.6.2 Properties of Carbon Nanoparticles

Bayda et al. (2017) claimed that CNPs attracted high interest of researchers because they are green materials with various properties such as tunable optical properties, high hydrophilicity, nontoxicity, excellent biocompatibility, able to serve as effective carriers for drugs and genes due to small size, effective in visual monitoring of controlled drug release, good cell permeability, high photostability, and high flexibility in terms of surface modification. Besides that, some other properties of CNPs include intrinsic electrochemical activity, large specific surface area, electrocatalytic properties, facilitated electron transfer, and outstanding electrical conductivity (Asadian et al., 2019). CNPs attracted more interest comparing to different types of carbon nanostructures such as CNTs, nanofibers, nanodiamonds, and so on due to their potential advantages which includes small size, simple and inexpensive synthetic routes, high aqueous solubility, and last but not least, their fluorescence property which enhance their use for cell imaging and their high cargo loading (Zeng et al., 2016).

2.2.6.3 Application of Carbon Nanoparticles as Electrochemical Sensor

CNPs have wide applications in areas such as bioimaging, drug delivery, sensors, optoelectronics, and photocatalysis (Zeng et al., 2016). An electrochemical sensor for bisphenol A consisting of multiwalled carbon nanotube (MW-CNT) and gold nanoparticle (AuNPs) composite modified glassy carbon electrode has been developed by Messaoud et al. (2017) with a LOD of 4 nM under optimum experimental conditions. The MW-CNT (0.05, 0.1, 0.2, 0.5 or 1.0)/GCE was prepared by casting 2 L of MWCNT (0.05, 0.1, 0.2, 0.5 or 1.0%) dispersion on the surface of the cleaned GCE with a micropipette and drying at room temperature for 24 h in order to obtain the best response of modified electrode.

Abo Hamad et al. (2016) and co-worker managed to design an electrochemical sensor *via* differential pulse voltammetry (DPV) technique through electrochemical oxidation of dopamine supported by metal nanoparticles such as ionic liquid-MWCNT-Au (IL-MW-CNT-Au) nanocomposite paste electrode. An impressive detection limit of 15 nM is obtained by the team. Inclusion of CNPs in MW-CNT and IL hybrid film on the surface of the electrode had improved the sensitivity towards dopamine oxidation successfully. This further supports the fact that carbon nanostructures can improve the LOD of the electrochemical sensor.

Maduraiveeran and Jin (2017) reported on sensitive electrochemical nitrobenzene (NB) sensor based on macro-/meso-porous carbon materials, which were fabricated by pyrolysis of the ionic liquid polymer pre-wrapped onto silicon dioxide microspheres obtaining a detection limit of 8 nM. The team further explained nanocomposite used was synthesized by one-step sol-gel and pyrolysis process. The resulting electrodes were successfully applied to the detection of Pb^{2+} and Cd^{2+} ions at concentration levels below 4 ppb in tap drinking water and wastewater systems.

Wang et al. (2014) proposed a three-dimensional (3D) macroporous carbon (3D-KSCs) derived from kenaf stem (KS) as a novel supporting material for the electrochemical sensing platform. A low detection limit of 0.05 mM was obtained for the electrochemical sensor produced. Meanwhile, Veerakumar et al. (2016) and co-workers reported on their research where palladium nanoparticles were deposited onto a porous activated carbon (derived from waste biomass feedstock) electrodes for the detection of toxic metal ions. The modified electrode was used as an electrode in the electrochemical sensor in detecting toxic heavy metal ions of Cd^{2+} , Pb^{2+} , Cu^{2+} , and Hg^{2+} .

2.2.6.4 Used of Chitosan Nanoparticles as Electrochemical Sensors

Muthusankar and Ragupathy (2018) foreseen that chitosan-based nanoparticles would attract great interest of researchers from various countries due to their rapid process, simple, and accurate measurements. Adhesive film-forming properties and numerous oxygen and nitrogen residues that plays a role in chemical modifications are the additional advantages of chitosan nanoparticles in the electrochemical sensor (De Lima et al., 2010; Solanki et al., 2011; Suginta et al., 2013; Abo Hamad et al., 2016). Chitosan nanoparticle with high surface area is valued as a tightly adhering and biocompatible porous polymer providing secure fixation of the enzyme to the biosensor surface *via* physical entrapment in the pores (Gomes et al., 2017). Chitosan nanoparticles supported electrochemical sensors have been used widely in detecting hepatitis B, various cancers, pregnancy, the iron content of blood, the food contaminant ochratoxin A, and diarrhea-triggering bacteria *Shigella flexneri* (Suginta et al., 2013).

Othman et al. (2018) reported covalent coupling of enzymes to chitosan nanoparticles electrode was achieved by cross-linking reagents such as glutaraldehyde, carbodiimides, N-hydroxysuccinimide, epichlorohydrin, or glyoxal. However, the

electrodeposition of chitosan nanoparticles and enzyme was reported by Ibrahim et al. (2015) to be the simpler method of chitosan-mediated enzyme electro-immobilization on biosensor comparing to a dispersion of nanoparticles *via* co-entrapment.

Drop-coated or electrodeposited chitosan films provided the elementary stage for surface immobilization *via* antibody or antigen producing a functional electrode coating (Ding et al., 2014). This is further proven when Anusha et al. (2014) and co-worker fabricated an enzymatic glucose biosensor *via* a simple drop-coat technique by introducing platinum (Pt) nanoparticles and chitosan (Ch) nanoparticles over the ZnO porous nanostructure. This combination results in good analytical performances with high sensitivity of $62.14 \text{ AmM}^{-1}\text{cm}^{-2}$ (Grenha, 2012).

On the other hand, Senel (2015) investigated an in-situ chemical synthesis method in fabricating glucose biosensor by using a film of chitosan-polypyrrole-gold nanoparticles; a sensitivity of $5.8 \times 10^{-4} \text{ ngmL}^{-1}$ is produced.

2.3 Electrochemical Detection

Electrochemical detection is an important method in monitoring sensing devices (Abo Hamad et al., 2016). The reaction is monitored electrochemically typically generates a measurable current (amperometry), charge accumulation or potential (potentiometry), and impedance. The ability to monitor sensing devices with a small volume of samples is the main advantage of electrochemical detection (Maduraiveeran & Jin, 2017). Electrochemical detection techniques are generally categorized into three main categories of measurement of current, potential and impedance.

2.3.1 Voltammetry and Amperometry

Voltammetry and amperometry techniques are characterized by applying a potential to a working electrode versus a reference electrode and measuring the current (Brajter-Toth

& Chambers, 2002; Button, 2015). The current is a peak which is proportional to the concentration of analyzed substance during electrolysis where redox reaction occurs at the working electrode. Voltammetry methods include linear sweep voltammetry, cyclic voltammetry, hydrodynamic voltammetry, differential pulse voltammetry, polarography, and stripping voltammetry (Maduraiveeran & Jin, 2017). The absence of a scanning potential distinguishes amperometry from voltammetry. The technique is implemented by stepping the potential directly to the desired value and then measuring the current or holding the potential and flowing samples across the electrode as in flow injection analysis (Beitollahi et al., 2019).

2.3.2 Impedance

Electrochemical Impedance Spectroscopy (EIS) is used widely in determining obstruction of the flow of electricity by new material or devices *via* AC signals through electrodes connected to the sample (JoVE Science Education Database, 2019). The data collected is then plotted by computer with the help of software for further analysis. EIS is well-known in detecting corrosion of metals, characterizing aging of food, measuring bacterial concentration in label-free biosensors, and study on ion mobility in batteries and supercapacitors (Button, 2015).

An AC voltage is applied during EIS at different frequencies and the electrical current is measured. Impedance (Z) is then calculated as the ratio of the frequency-dependent potential (E) to the frequency-dependent current (I) (Button, 2015). A semi-circle Nyquist plot is generated from the frequency response to the electrical impedance where an imaginary component on the y-axis and the real component on the x-axis. An example of a simple Nyquist plot is shown in Figure 2.2.

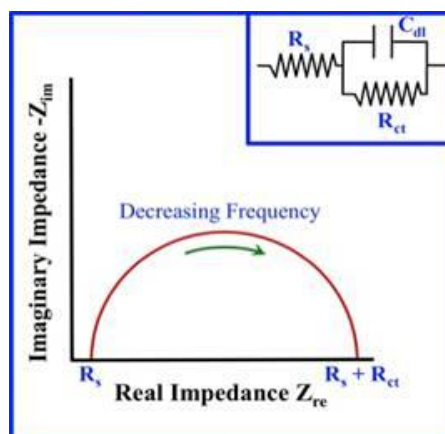


Figure 2.2: Image of a simple Nyquist plot.

2.4 Electrodes

Mindtouch® (2019) defined electrode as a metal where its surface where redox reaction occurs when current enters (*via* anode) and leaves (*via* cathode) the electrolyte in an electrochemical cell. Atom becoming positively charged ions when an anode donates electrons from the electrolyte mixture while atom which receives electrons forming a negatively charged ion (Hayat & Marty, 2014). Three electrode systems are made up of a working electrode, a reference electrode, and a counter electrode which carries an important role in voltammetry, respectively. The function of each electrode is shown in Table 2.1 while conventional electrode such as standard hydrogen electrode, calomel electrode, silver-silver chloride electrode, and screen-printed carbon electrode was discussed in the later section.

Table 2.1: Function of electrode (Hayat & Marty, 2014).

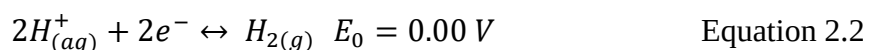
Electrode	Function
Reference	It has a stable and well-known electrode potential and is used as a half-cell in an electrochemical cell to ensure the electrode potential of the other half-cell can be discovered.

Table 2.2 continued

Counter	Making a connection to the electrolyte in applying current to the working electrode without passing through a reference cell.
Working	Transfer electrons to and from the substances that are present.

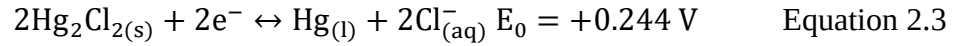
2.4.1 Conventional Electrodes

The standard hydrogen electrode (SHE) has been chosen as the reference electrode for all measurements of half-cell potential reaction as the electrode potential of the hydrogen electrode is fixed at 0.00 V (Matsui et al., 2015). However, SHE is difficult to operate because to maintain constant stream of hydrogen at 1 atm needs constant adjustment and evolves gases (Biegler & Woods, 1973). SHE is made up of bubbling hydrogen at a pressure of 1 atm over the surface of platinum coated with platinum oxide electrode which is immersed in an acid solution of H^+ ion concentration of 1 M (Mindtouch®, 2019). Matsui et al. (2015) described the role of platinum oxide is to facilitate the adsorption of hydrogen gas on its surface while catalyze the equilibrium between hydrogen gas and H^+ ion based on the redox half-equation at Equation 2.2.

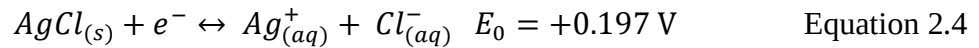


Standard calomel electrode (SCE) consists of a mercury and mercury-chloride molecules placed in a tube with a porous salt bridge which allows flows of ions in order to complete the circuit (Yang et al., 2017). SCE is more convenient compared to SHE as it does not involve gases. According to Vera (2015), the standard electrode potential of SCE is +0.244 V with respect to SHE when mercury and solid mercury (I) chloride attached to a rod that is immersed in a saturated potassium chloride solution (Wikipedia, 2020). A

saturated solution is important as it will lower the voltage referring to SCE and allows the exchange of chlorine ions (supplied by saturated potassium chloride solution) (Grzybowski, 1958; Yang et al., 2017). The half-equation of a cell is as follows.



Silver- silver chloride electrode is a thin tube that consists of potassium chloride precipitate and silver chloride solution (Fajac et al., 1998 Mindtouch®, 2019). It has been used widely compared to SPCE as it is inexpensive, non-toxic and does not contain mercury (Yang et al., 2017). Vera (2015) mentioned the standard electrode potential of silver-silver chloride electrode is +0.197 V with respect to SHE when a solid silver wire code in silver chloride and is placed in a tube of potassium chloride and silver chloride solution which in turn allows the formation of ions during the flow in and out of electrons from the electrode system (Wikipedia, 2020). The half-equation of the cell is as shown below.



2.4.2 Screen-Printed Carbon Electrode (SPCE)

Nanotechnology has enabled the fabrication of screen-printed devices using stencils, ink, and squeeze blades on bendable substrates (Yamanaka et al., 2016). Screen-printed carbon electrodes (SPCE) are low cost disposable electrochemical devices that are fabricated in bulk, highly reproducible, small and easy to operate a device, do not require experienced technicians, and can be used immediately without any pre-treatment needed. Akhtar and Jean (2014) mentioned SPCE as a well-established technique as all the three electrodes namely counter, reference, and working electrodes are printed on the substrate surface and ready to be used. SPCE is converted to a detectable signal which will then integrate by a

transducer (Metters et al., 2011; Geng et al., 2016). The image of SPCE is shown in Figure 2.3.

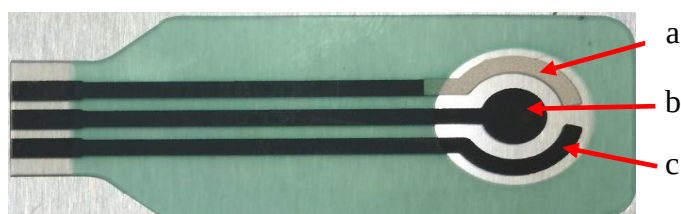


Figure 2.3: Image of SPCE, (a) Reference electrode, (b) Working electrode, and (c) Counter electrode.

Abo Hamad et al. (2016) successfully drop-coat a mixture of vortex-assisted ionic liquid dispersive liquid-liquid microextraction (IL-DLLME) with microvolume back extraction on a (AuNPs) modified SPCE to determine Hg(II) in water sample and non-digested urine samples with LOD approximately 0.2 ngmL^{-1} and $0.5\text{--}1.5 \text{ ngmL}^{-1}$ respectively.

2.5 Immobilization Procedure

Indirect immobilization of enzyme is to confine enzyme to support (inert polymers and inorganic materials) different from the one for substrates and products; while direct immobilization happens when the whole cell is immobilized (Datta et al., 2013; Feng et al., 2018). An ideal matrix must have the characteristics of inert, high physical strength, stable, regenerable, ability to increase enzyme specificity and reduce product inhibition, nonspecific adsorption, and microbial contamination, and affordable (Carter & Shieh, 2015).

Nguyen et al. (2019) reported that it is important for recognition elements in a biosensor to immobilize onto the surface of transducers so that they can interact with target molecules without adding reagents into the sample solution which will then cause a physicochemical change onto transducer before measurable signal obtained to determine the amount of analyte. Both direct immobilizations of recognition element used in biocatalytic

biosensor and indirect immobilization of recognition element used in affinity biosensor will be discussed in Section 2.5.1 and Section 2.5.2 respectively.

2.5.1 Used Direct Immobilization on Electrode Surface

Biomolecules are directly immobilized on the electrode surface via physical or chemical modification in order to improve the sensitivity of materials and probes in detecting recognition elements (Liu et al., 2018). This method has led to the research and production of portable enzyme sensors. Geng et al. (2016) developed a multiplexed electrochemical sandwich-type immunoassay for the detection of carcinoembryonic antigen (CEA) and α -fetoprotein (AFP). The group prepared the immunosensor by direct immobilizing antibodies on chitosan modified SPCE with LODs of $3.5 \times 10^{-5} \text{ ngmL}^{-1}$ and $3.9 \times 10^{-5} \text{ ngmL}^{-1}$ for CEA and AFP, respectively (Arduini et al., 2016). Moreover, Gupta et al. (2014) successfully developed electrochemical immunosensor for the detection of prostate-specific antigen (PSA). They explored the detection of PSA by immobilizing horseradish peroxidase (HRP)-labelled antibody (Ab2) functionalized with gold nanoparticles (HRP-Ab2/GNPs) onto the surface of immunosensor obtaining a LOD of $4.6 \times 10^{-4} \text{ ngmL}^{-1}$.

2.5.2 Used Other Solid Supports as Carrier

Recognition element is immobilized on a carrier and then install on the electrode surface on the probe of the sensor so that the sensitive part is independent of the converter and then prolong the service life of converter which is then used in long time monitoring probe (Liu et al., 2018). This method leads to the research and production of enzyme sensors used in processing online analysis and process control in industry. Pandiaraj et al. (2014) reported on their success in designing an immunosensor with LOD of 2 nM for the detection of cytochrome by indirect immobilization of monoclonal antibody *via* self-assembly method onto the gold nanoparticle (GNPs) surface and then was electrodeposited onto the surface of

polypyrrole (PPy) matrix. Meanwhile, Ravalli et al. (2013) studied immunosensor for the detection of cancer antigen 125 (CA 125). The method used by the group was self-assembled monolayer (SAM) on the surface of GNPs and then electrodeposited on SPEs using a thiolic compound (11-mercaptoundecanoic acid) and successfully obtained a LOD of 6.7×10^{-4} ngmL⁻¹ (Ravalli et al., 2013).

2.6 Japanese Encephalitis Virus (JEV)

2.6.1 Overview of Japanese Encephalitis Cases

JE is a mosquito (*Culex tritaeniorhynchus*) - borne disease which has caused nearly 68,000 clinical cases every year in Asia, with up to 30% deaths based on a report by Meenu and Xu, (2019). Humans are dead-end hosts of JEV as they are not able to develop a sufficiency level of viremia to re-infect other mosquitoes, hence transmission among humans is almost impossible (Zhang & Hoshino, 2018). Amicizia et al. (2018) reported about 34,000 cases per year occurred in China alone as a quality vaccination program was not emphasized properly. On the other hand, Japan, the Republic of Korea and Taiwan are “high-incidence” countries, however, their JE incidence was brought to a limit of 0.003 per 100,000 in the overall population due to efficient vaccination program began in Japan in 1954, in Taiwan in 1968, and in Korea in 1970 (Heffelfinger et al., 2017). Vietnam, Myanmar, Cambodia, Malaysia, and Nepal still experiencing occasional epidemics of JE (Kumar et al., 2018). JE is an inflammation of the brain which will cause symptoms like headache, fever, confusion, seizures and may eventually lead to fatality (Erlanger et al., 2009).

2.6.2 The Conventional Diagnostic Methods for JEV Detection

JEV is a member of the Flaviviridae family which contains a single positive 11-kb RNA genome with three structural proteins and seven non-structural proteins with E protein

as the major immunogenic protein was first discovered by Sumiyoshi et al. (1987). Sumiyoshi and co-worker first discovered that E protein act as the main protein in developing vaccines and diagnosis method due to its ability in inducing neutral antibodies. Four conventional diagnostic methods namely enzyme-linked immunosorbent assay (ELISA), plaque reduction neutralization test, reverse transcription-polymerase chain reaction (RT-PCR), and virus isolation of JE will be discussed in Section 2.6.2.1 to Section 2.6.2.4.

2.6.2.1 Enzyme-linked Immunosorbent Assays (ELISA)

Enzyme-linked immunosorbent assay (ELISA) provides the advantages of high specificity, simple, stable, and rapid analysis and hence becoming a well-known analytical method in detecting JEV *via* specific interaction between an antibody and its antigen (Hobson-Peters, 2012; Chen et al., 2018). ELISA is commonly used to measure the concentration of a soluble protein-coated to an assay plate *via* a three-step detection process and a colour changes proportional to the concentration of protein bound to the plate will be observed (Liddell, 2001a; Amicizia et al., 2018). Chen et al. (2018) established an antigen-capture ELISA to detect the JEV antigen. 60 clinical samples were tested, and the results demonstrated high sensitivity and specificity ELISA was established. 14 samples showed a positive result with a coincidence rate of 70%, and 46 displayed negative results with a coincidence rate of 100%. On the other hand, Feng et al. (2018) developed ELISA detecting NS1 antibodies in equine sera and proven ELISA is sensitive to detect subclinical infections in vaccinated equine populations.

2.6.2.2 Plaque Reduction Neutralisation Test

Plaque reduction neutralization test (PRNT) is used to identify and confirm arbovirus infection in diagnostic laboratories as well as monitor immunological protection in vaccine

recipients (Johnson et al., 2009; Cha et al., 2014). Wild-type viruses such as Japanese encephalitis virus (JEV) used in the PRNT may be difficult to grow and plaque titrate and JEV are categorized under the jurisdiction of the select-agent program which can only be used in approved laboratories (Gayen et al., 2017). According to Cha et al. (2014), PRNT is the most reliable method in the evaluation of the efficacy of the vaccine or patient diagnosis despite its 5 to 7 days of turnaround time for most flaviviruses and lead to difficulties on quality control. The group further explained PRNT show advantageous as strong cross-reactions occur between antibodies induced by flavivirus infections and hence enabled PRNT to identify specific pathogen accurately. PRNT was able to test vaccine efficacy for the JE vaccine with a small number of serum samples in 24 well plates but the disadvantage of PRNT is that it needs special technical operators and only a small number of samples can be tested (Gupta et al., 2014).

2.6.2.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reverse transcription-polymerase chain reaction (RT-PCR) uses enzyme reverse transcriptase mRNA as a starting template to produce a complementary single-stranded DNA called cDNA (Bharucha et al., 2018). cDNA is then converted into DNA molecules which act as the template for PCR reaction with the help of DNA polymerase. RT-PCR value is then used to determine if an mRNA species is present in a sample or to clone a cDNA sequence for a subsequent experiment later (Carter & Shieh, 2015). Bharucha et al. (2018) and co-worker demonstrated a one-step RT-PCR assay developed that allows the simultaneous detection of West Nile Virus (WNV) and JEV. A diagnosis of approximately 3.5 h including nucleic acid extraction, amplification, and gel electrophoresis. Although RT-PCR gives a rapid, sensitive, and specific result, it is not widely used in JE diagnosis because

it requires experienced technicians and specialized laboratory equipment. RT-PCR has not able to detect a large number of samples and a level of JEV (Amicizia et al., 2018).

2.6.2.4 Virus Isolation

Viruses are different from bacteria; they are able to grow on artificial nutrient medium but still need to be cultured and grown on infected host cells and then separate the virions in the liquid medium from the host cells by centrifugation or filtration. Virus isolation defined by Morley and Pusterla (2013) as a method of diagnosed viral infection. However, isolation techniques were found to be insensitive as a diagnostic test as culture techniques need a week or more for completion and require specialized laboratory equipment and skills.

2.6.3 Electrochemical Immunosensors for JEV Detection

An electrochemical cell consists of an electrochemical sensor with two or three electrodes (a working electrode, a reference electrode and a counter electrode) (Rezaei et al., 2016). Both two and three-electrode systems are used in sensors, but two electrodes are chosen over three-electrode system for disposable sensors due to its long-term stability of the reference and the hence reduced the cost (Ravi, 2019). Difficulties faced in the detection of JEV infection due to the short duration of viremia and the asymptomatic nature of JE infection but detection of JEV using conventional method took the longer time (Mansfield et al., 2017). Hence, this leads to the growing interest of researchers on electrochemical immunosensor for JEV detection in a shorter period with high accuracy which will be discussed further in Section 2.6.3.1 and Section 2.6.3.2.

2.6.3.1 Application of CNPs-SPCE Electrochemical Biosensor Strip

Carbon electrodes draw tremendous interest in electro-analysis more than three decades ago due to their low background current, wide potential range, chemical inertness

and suitability for various sensing and detection applications (Akhter, 2017). Fabrication of disposable electrochemical immunosensor strip for the detection of JEV using CNP-modified SPCE was reported by Chin et al. (2017). They prepared CNPs from starch nanoparticles and deposited on SPCE before functionalized with 3-aminopropyl triethoxysilane followed by immobilization of the JEV antibody on it. A LOD of 2 ng mL^{-1} has been reported by the same group.

2.6.3.2 Application of Au-CNPs-SPCE Electrochemical Biosensor Strip

Hybrid gold-carbon nanoparticles (Au-CNPs) modified SPCE electrochemical biosensor strip can be fabricated easily *via* simple self-assembly method (SAM) and affinity property of gold leads to the increase in interest of researchers in investigating the applicability of gold SPCE in electrochemical sensors (Du et al., 2007; Pan et al., 2015). Geng et al. (2016) discovered a high sensitivity, specificity and disposable electrochemical impedance biosensor by Au-CNPs modified SPCE to detect JEV. HRP-labelled second antibody was linked to the biosensor *via* sandwich-type biosensor in order to amplify the signal with LOD of 167 pfum L^{-1} . Apart from that, Asadian et al. (2019) also reported on their success in fabricating a sensitive detection of AFP using a similar method as Geng and co-workers. However, Asadian and co-worker used HRP-anti-AFP-ISNGs as trace labels and obtained LOD as low as 10 pg mL^{-1} . The illustration diagram of their research is as shown in Figure 2.4.

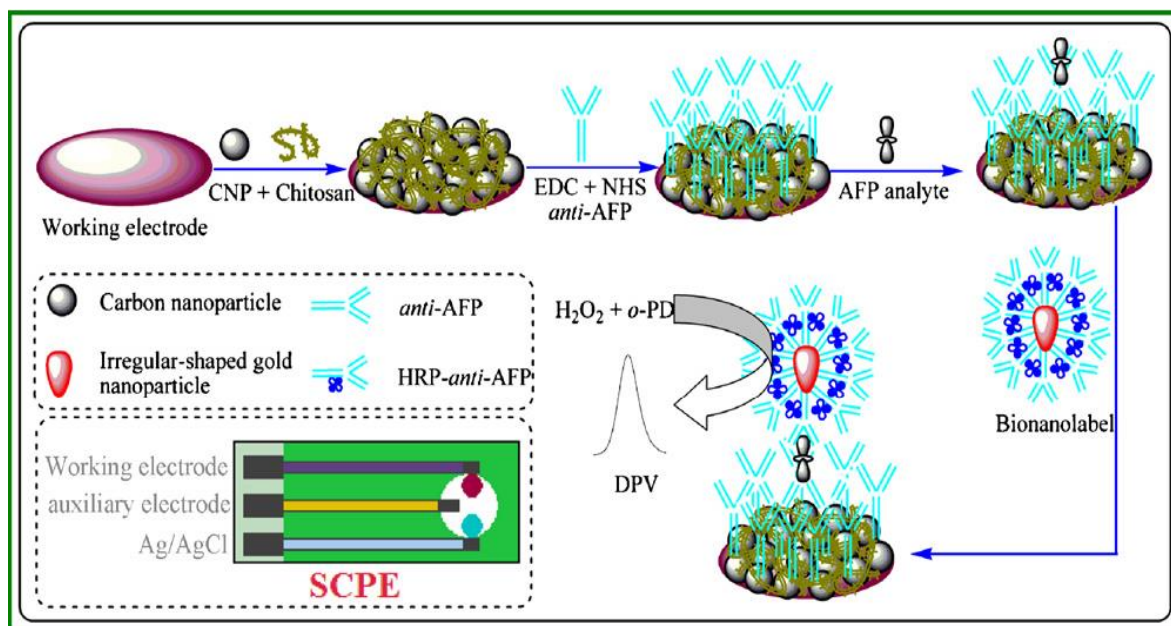


Figure 2.4: Fabrication of sensitive detection of AFP (Asadian et al., 2019).

In generally, the focus for this work is to fabricate and characterised a CNPs based electrochemical biosensor for JEV detection. From the current literatures that had been discussed in this chapter, although numerous researches on biosensor had been reported worldwide, but the enhancement work is still needed to produce a more reliable, high sensitivity and rapid point-of-care biosensor. Thus, significant efforts have been devoted to the facile synthesis of different types of carbon-based nanoparticles with controllable morphology and tuneable dimensions, and their subsequent important role in enhancing their properties in electrochemical immunosensor for JEV detection which will present in detailed in Chapter 3 and Chapter 4. However, as Au-NPs well known reported with its obvious sensitivity toward biological binding, therefore, further investigation for the enhancement of the biosensor by using the CNPs synthesized in Chapter 4 and Au-CNPs to fabricate hybrid (Au-CNPs-SPCE) electrochemical biosensor strip is carried out in Chapter 5.

CHAPTER 3

SYNTHESIS AND CHARACTERISATION OF VARIOUS CARBON-BASED NANOPARTICLES (CNPs) FOR BIOSENSING APPLICATION

3.1 Introduction

Nanoparticles with the size ranged between 1 to 100 nm exist in amorphous or crystalline form in which its surface normally acts as a carrier for liquid (Sharma et al., 2019a). Khan et al. (2019) reported on the unique physical and chemical properties of nanoparticles due to their high surface area, size, shape, and structure which later affects its reactivity, toughness and other properties and hence becoming popular among researchers in different fields including biomedical, biosensor, catalysis, imaging, energy-based research, and environmental research (Lauter et al., 2016; Sharma et al., 2016).

Carbon nanomaterials from zero-dimension (0D) to three-dimension (3D) have been adapted for the development of electrochemical biosensors. Carbon nanomaterials such as carbon nanotubes (CNTs), graphene and its derivatives offer diverse advantages in electrochemical biosensing application especially with high performance in terms of field-effect mobility and sensitivity (Angione et al., 2011). However, carbon nanomaterials with large surface-to-volume ratio possess the ability to acquire and distinguish electrical signals before and after hybridization of biological elements (Tiwari et al., 2016) and the high electrical conductivity and other intriguing physics in carbon caused by the delocalized π electron network facilitate rapid electron transfer kinetics from the transducer surface to the electrochemical medium (Pumera et al., 2010). In addition, the electrochemical properties of carbon nanomaterials also enable smooth electron transduction of electrochemical responses (Muniandy et al., 2019).

Recently, CNPs have attracted numerous researchers to study their potential nanoscience and nanotechnology potential comparing to other nanoparticles due to their special characteristics especially their optical and electrochemical properties which turn them into potential candidates for bioimaging, biosensing, drug delivery, and photodynamic applications (Asadian et al., 2019) and most importantly CNPs are easier to synthesize, environmental friendly, energy-saving, and low cost (Sharma et al., 2016). The roles of carbon materials in electrochemical immunosensors have gradually expanded from electrode materials to building blocks (Fang & Wang, 2013; Wu et al., 2013; Wang & Dai, 2015). Although the properties and performance for different types of CNPs may vary, their common features render them extremely attractive for the construction of electrochemical immunosensors as detailed below.

- 1) Electrochemical activity (Ambrosi et al., 2014). Certain CNPs and their derivatives can exhibit electrochemical responses due to their intrinsic or endowed electrochemical activity.
- 2) Electrical conductivity (Fang & Wang, 2015; Wang & Dai, 2015). The high electrical conductivity of CNPs enables them to serve as an ideal medium for the transfer of electron in electrochemical immunosensors.
- 3) Higher total surface area (Peigney et al., 2001). Because of smaller size resulting in large surface area of CNPs, additional required components can be assembled in electrochemical immunosensors.
- 4) Ease of functionalization (Yang et al., 2013). CNPs can be easily modified, which will enhance the functions of CNPs in electrochemical immunosensors.
- 5) Biocompatibility (Saito et al., 2014). The biocompatibility of CNPs facilitates applications of carbon nanoparticle-based devices in biological fields. Because of these attractive properties, CNPs can act either as nanoprobes, relying on their dominant electrochemical properties or as nanocarriers relying on their other multiple properties. Thus, with the application of CNPs in electroanalytical assays, analytical performances can be substantially

improved and able to produce a more reliable, rapid and high sensitivity electrochemical biosensor.

CNPs can be synthesized from various types of precursor namely cellulose, chitosan, starch, ascorbic acid, cellulose is a skeleton component of most plants and are one of the popular choices of precursor used in preparing CNPs due to its abundant availability, renewability, low cost, easy to prepare, and minimum impact towards the environment (Oh et al., 2019). Oh et al. (2019) and the groups reported that cellulose fibre has severe unique properties which include hierarchical structures, presence of hydroxyl group, flexibility, and excellent mechanical properties even after high-temperature carbonisation. Gaminian et al. (2019) proposed that nanosized cellulose fibre having high surface area to volume ratio will improve the properties of CNPs produce and hence they prepared CNPs from cellulose using the sputtering method. However, they later found out cellulose with poor solubility with no possibility of melting might lead to certain limitations of using cellulose in the preparation of CNPs.

Chitosan derived from chitin which is the second most abundant polysaccharide after cellulose (Liu et al., 2016; Yadav et al., 2019). Chitin can be found easily in the exoskeletons of crabs, shrimps, insects and other anthropods (Muthusankar et al., 2018). The remarkable biological properties related to biocompatibility, biodegradability, antibacterial activity, and non-toxicity successfully attracted extreme interest from various researchers. Shafiei et al. (2019) mentioned chitosan CNPs are stabilising agents for enzyme immobilisation due to its excellent film-forming ability, mechanical strength, cost-effectiveness, and finally the amino groups of NH_2 in chitosan provides hydrophilic chain that results to it compatible with biomolecules.

As there are many types of carbon-based precursor can be used as a substrate for the preparation of CNPs and it is crucial to investigate the most promising precursor in the preparation of CNPs for biosensing application to fabricate the most reliable, economical, rapid and high sensitivity biosensor. Therefore, in this study, different types of precursor including carboxymethylcellulose, starch, citric acid, fibrous cellulose, ascorbic acid, and chitosan based CNPs were prepared *via* recently popular and time saving reflux with an acid method. The morphology, size and electrical conductivity of the CNPs formed were characterised and the CNPs that showed a high superior performance of electronic properties will further optimize and undergoes functionalisation in the next chapter for enhance its biosensing properties.

3.2 Materials and Methods

3.2.1 Chemicals

All chemicals were of reagent grade and used without further purification. Sodium tripolyphosphate (TPP) of technical grade (85%), acetic acid was from HmbG Chemicals (Hamburg, Germany), chitosan powder with molecular weight of 100–300 kDa and carboxymethylcellulose powder with low viscosity were purchased from Acros Organics (New Jersey, USA), sulphuric acid of technical grade (95%) was purchased from J.T.Baker (Thailand), phosphate buffer saline (PBS, pH 7) and citric acid powder were obtained from Merck (Germany), ascorbic acid and starch soluble were supplied by Sigma-Aldrich, fibrous cellulose powder CF11 was purchased from Whatman, acetic acid glacial ($C_2H_4O_2$), potassium ferricyanide ($K_3[Fe(CN)_6]$) and potassium ferrocyanide ($K_4[Fe(CN)_6]$) were obtained from Bendosen (Bendosen Laboratory Chemicals, Bendosen, Norway). Screen-printed carbon electrodes (SPCE) with a carbon-based working electrode, carbon-based counter electrode, and a silver/silver chloride (Ag/AgCl) reference electrode were purchased

from a local vendor Rapid Genesis Sdn Bhd. Ultra-pure water (18.2 MΩ.cm) was obtained from a water purifying system (ELGA Model: Ultra Genetic).

3.2.2 Synthesis of Preformed Nanoparticles from Different Types of Precursor

The preformed nanoparticles from different types of precursor (carboxymethylcellulose (CMC), starch, citric acid, fibrous cellulose, chitosan, and ascorbic acid) were synthesized based on previous reported simple nanoprecipitation method (Chin et al., 2011; Yazid et al., 2013; Chin et al., 2017). In general, nanoparticles of carboxymethylcellulose (CMC), starch, citric acid, fibrous cellulose, chitosan, and ascorbic acid were prepared by adding dropwise of the solution from different types of precursor to excess absolute ethanol. The nanoparticles suspension was collected by centrifuge for 30 min at 13,500 rpm and the sample were subsequently converted to CNPs by reflux with concentrated sulphuric acid, H₂SO₄.

3.2.3 Synthesis of Carbon Nanoparticles (CNPs) from Different Types of Precursor

For the dehydration reaction with concentrated sulphuric acid, H₂SO₄, 2 g of the preformed nanoparticles were dispersed in 5 mL of ultrapure water, followed by the addition of 8 mL of concentrated sulphuric acid H₂SO₄. The resulting mixture was stirred for 40 min and then 40 mL of ultrapure water was added. The black carbonaceous nanoparticles produced were then collected *via* centrifugation (13,500 rpm), rinsed and dried in a vacuum desiccator for 1 day.

3.2.4 Instrumentation

The morphology of samples was observed using a field emission scanning electron microscopy (FESEM) (JSM – IT500 HR) and scanning electron microscopy (SEM) (JOEL, JSM-6390 LA). Samples were deposited on aluminium stubs, dried at room temperature,

and coated with a thin layer of platinum using JEOL/JFC-1600 Auto Fine Coater prior to FESEM and SEM analysis. The mean diameter (particles size) of the CNPs obtained from different types of precursor were determined using the Smile View Software by measuring the diameter of at least 50 discrete and spherical nanoparticles as observed in the SEM and FESEM micrograph. FTIR spectra of samples for CNPs prepared by various temperatures were obtained from KBr sample pellets in the range of 400–4000 cm^{-1} on a FTIR spectrometer (Thermo Scientific, Nicolet iS10). X-ray diffraction (XRD) (BRUKER/D8 ADVANCE) from UKM was used to study the structural properties of chitosan CNPs.

3.2.5 Electrochemical Characterization

The electrochemical properties performance for various types of CNPs were characterised by (i) CV in the presence of 10 mM $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (1:1) as redox probes and 0.1 M KCl at pH ~7.4 and the potential was cycled between -1.3 V and $+1.0$ V (vs Ag/AgCl) at a scan rate of 50.0 mVs^{-1} and (ii) EIS measurements were also carried out in the presence of 10.0 mM of $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (pH ~7.4) containing 0.1 M KCl in order to further investigate the effects of electron transfer on the electrode surface. CV and EIS measurements were performed on a Potentiostat (Princeton Applied Research, PARSTAT 2263) interfaced with a computer installed with the Power Suite software package was used for the electrochemical evaluation of the CNPs synthesized from various precursor.

3.3 Results and Discussion

3.3.1 Size and Morphology of Carbon Nanoparticles

As we know, nanoparticles with the size ranged between 1 to 100 nm normally exists in amorphous or crystalline form and amorphous particles usually adopt a spherical shape

(due to their microstructural isotropy), whereas the shape of anisotropic microcrystalline whiskers corresponds to their crystal habit. At the small end of the size range, nanoparticles are often referred to as clusters. In this section, six types of carbon-based precursor namely carboxymethylcellulose, starch, fibrous cellulose, citric acid, ascorbic acid and chitosan were chosen to synthesize CNPs *via* refluxing with acid methods. As reported by Khan et al. (2019), the unique physical properties of nanoparticles due to their size, shape, and structure that may affects its reactivity, toughness, conductivity, sensitivity as well as other properties. Therefore, the size and morphology of the different types of CNPs synthesized from different precursor were characterised by using FESEM and the particles size of the CNPs were determined using the Smile View Software.

Figure 3.1 (A) to (F) showed FESEM micrographs of CNPs of different types of precursors namely carboxymethylcellulose, starch, fibrous cellulose, citric acid, ascorbic acid, and chitosan obtained from refluxing with acid methods. From the FESEM micrograph and Smile View software analysis, although all the carbon nanoparticles exist in a spherical shape (Supriya et al., 2019), but it is clearly showed that different types of the precursor may lead to the various sizes of particles ranging from ± 89 nm to ± 156 nm. Among all the CNPs that had been synthesize in this studies, chitosan CNPs showed the smallest particles size (89 nm) followed by starch soluble, fibrous cellulose, ascorbic acid, carboxymethyl cellulose while citric acid CNPs was reported with the biggest size of 156 nm. Detailed for all the CNPs particles size from different types of precursor with its error bar were reported in Figure 3.2. Meanwhile, chitosan CNPs with the smallest size may have the higher potential in biosensing as the smaller the size of the CNPs, the larger the total surface area that enables surface functionalization and immobilisation of biological molecules, high electron mobility and excellent conductivity (Muniandy et al., 2019).

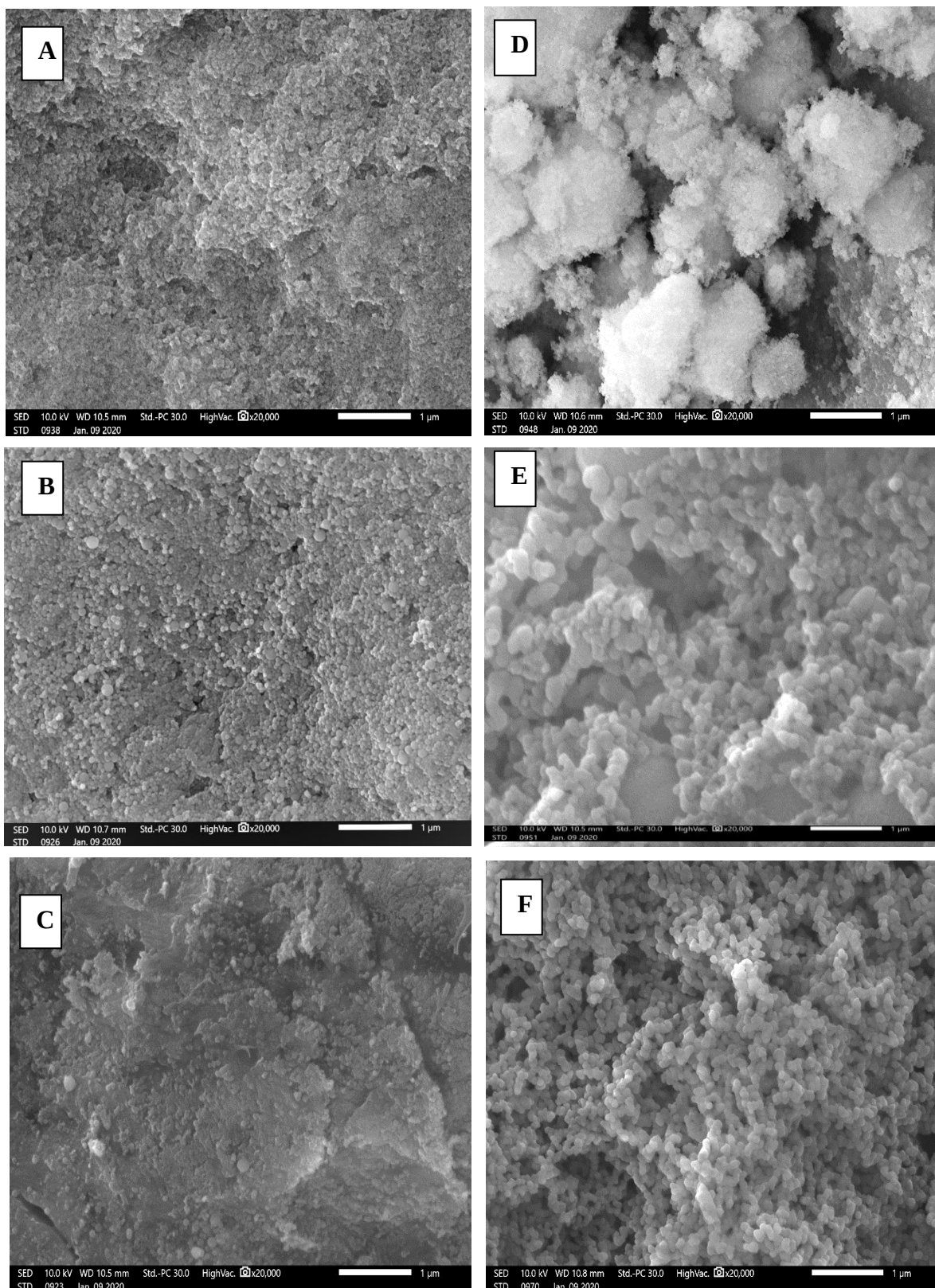


Figure 3.1: FESEM micrographs of CNPs from different types of precursor (A) carboxymethyl cellulose, (B) starch, (C) fibrous cellulose, (D) citric acid, (E) ascorbic acid and (F) chitosan.

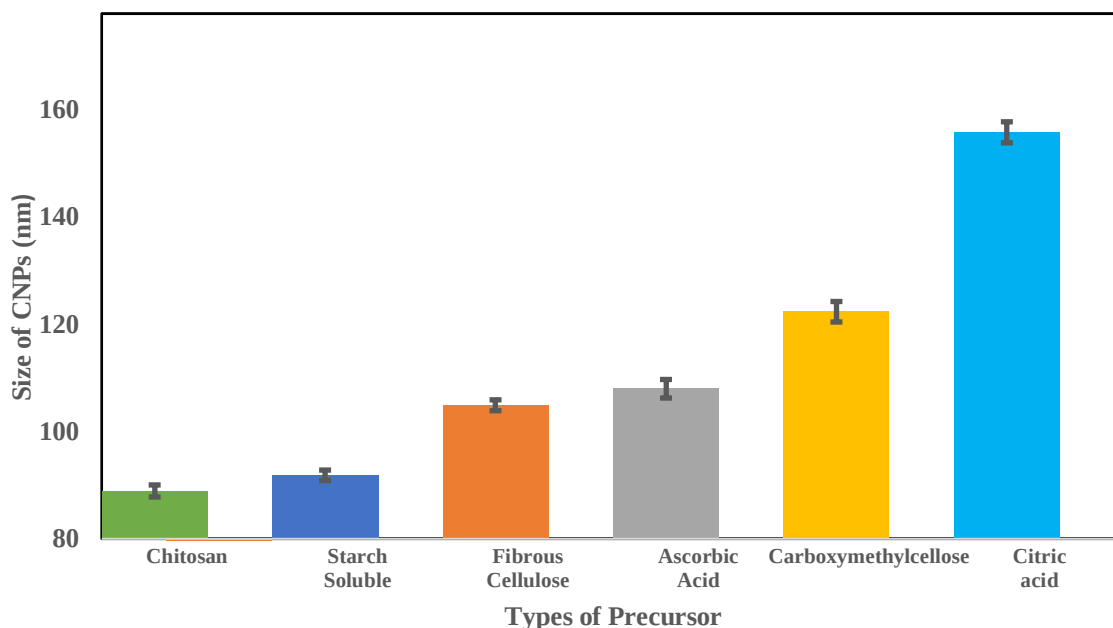


Figure 3.2: Bar chart showed the particles size and error bar (calculated from standard deviation of particles size for 50 discrete and spherical nanoparticles) of CNPs synthesized from different types of precursor.

3.3.2 Electrochemical Characterization of Carbon Nanoparticles

3.3.2.1 Cyclic Voltammetry (CV)

Approximately 10 μL of the CNPs prepared from different types of precursor were dropped coated onto the working electrode of the SPCE strip and incubated for 48 hours at 37 $^{\circ}\text{C}$ *via* physically binding mechanism and was characterized by cyclic voltammetry in the presence of 10 mM $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (1:1) as redox probes and 0.1 M KCl at pH ~ 7.4 . Cyclic voltammograms in Figure 3.3 showed that the CNPs from different types of precursors (carboxymethylcellulose (CMC), starch, citric acid, fibrous cellulose, chitosan and ascorbic acid) coated SPCE strip exhibited higher both anodic and cathodic current density than bare SPCE strip. The presence of CNPs led to an increase in the conductivity and effective electroactive surface area of SPCE. Of all the carbon-based materials used in this study, chitosan CNPs showed the highest magnitude of both anodic and cathodic current follow by starch CNPs, fibrous cellulose CNPs, ascorbic acid CNPs,

carboxymethylcellulose CNPs and citric acid CNPs exhibited the smallest changes of the magnitude for both anodic and cathodic current. SPCE strip coated with chitosan CNPs and starch CNPs exhibit a higher electrical conductivity when characterized electrochemically by using cyclic voltammetry and the finding showed an inversely proportional relationship with the size of the particles where the smaller the size of the CNPs, the larger the total surface area resulting more surface area of CNPs exposed for the electron transfer and lead to higher electrical conductivity (Fang & Wang, 2013; Wang & Dai, 2015). In addition, higher electrical conductivity enables they serve as an ideal medium for the transfer of electron in electrochemical biosensor.

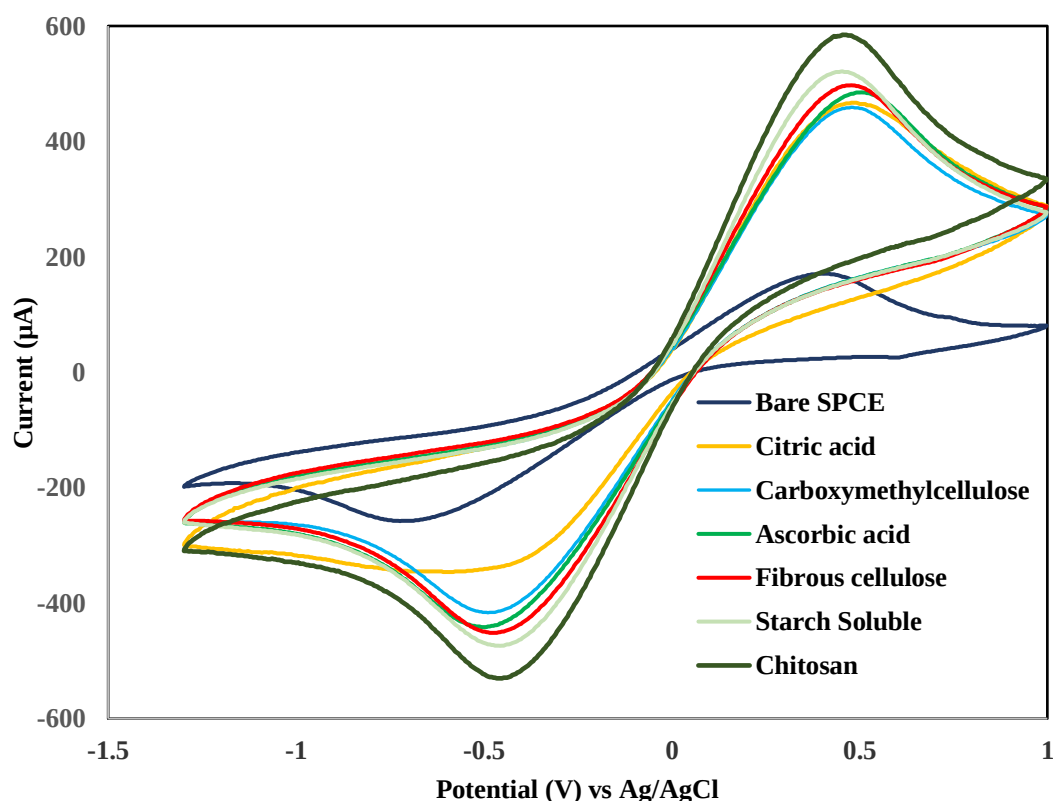


Figure 3.3: Voltammograms of bare SPCE strip and CNPs from different types of precursor modified SPCE strip.

3.3.2.2 Electrochemical Impedance Spectroscopy (EIS)

EIS measurements were carried out in the presence of 10.0 mM of $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (pH ~ 7.4) containing 0.1 M KCl in order to further investigate the effects of electron transfer on the electrode surface. All EIS data were simulated by Randle's Equivalent Circuit model as shown in the inset of Figure 3.4. This circuit includes the uncompensated resistance of the solution phase (R_s), Warburg impedance (Z_w) resulting from the diffusion of ions from bulk electrolyte to electrode surface, double-layer capacitance (C_{dl}), and electron-transfer resistance (R_{et}) which is inversely proportional to the rate of electron transfer (Piermarini et al., 2013). The nyquist plot from Figure 3.4 that carried out by using EIS showed that the findings are matching with the earlier findings that reported in Section 3.3.2.1 analyse by using CV. The deposition of CNPs onto the SPCE electrode surface had resulted in a significant decrease of R_{et} from 884 ohm (bare SPCE Strip) to lower ranging from 612 to 831 ohm (CNPs coated SPCE Strip) due to the lower resistance of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ electron transfer process. However, as observed in Figure 3.4, the nyquist plot of CNPs coated SPCE strip displayed a smaller semicircle with a smaller diameter than that of bare SPCE strip which mean the CNPs coated SPCE strip had exhibited a lower electron transfer resistance and faster electron transfer rates at the electrode surface as compared to bare SPCE strip. These superior electronic properties of CNPs make them extremely sensitive to small changes in charges resulting from the binding of antigen to the transducers and causes a noticeable read-out in electronic-sensors.

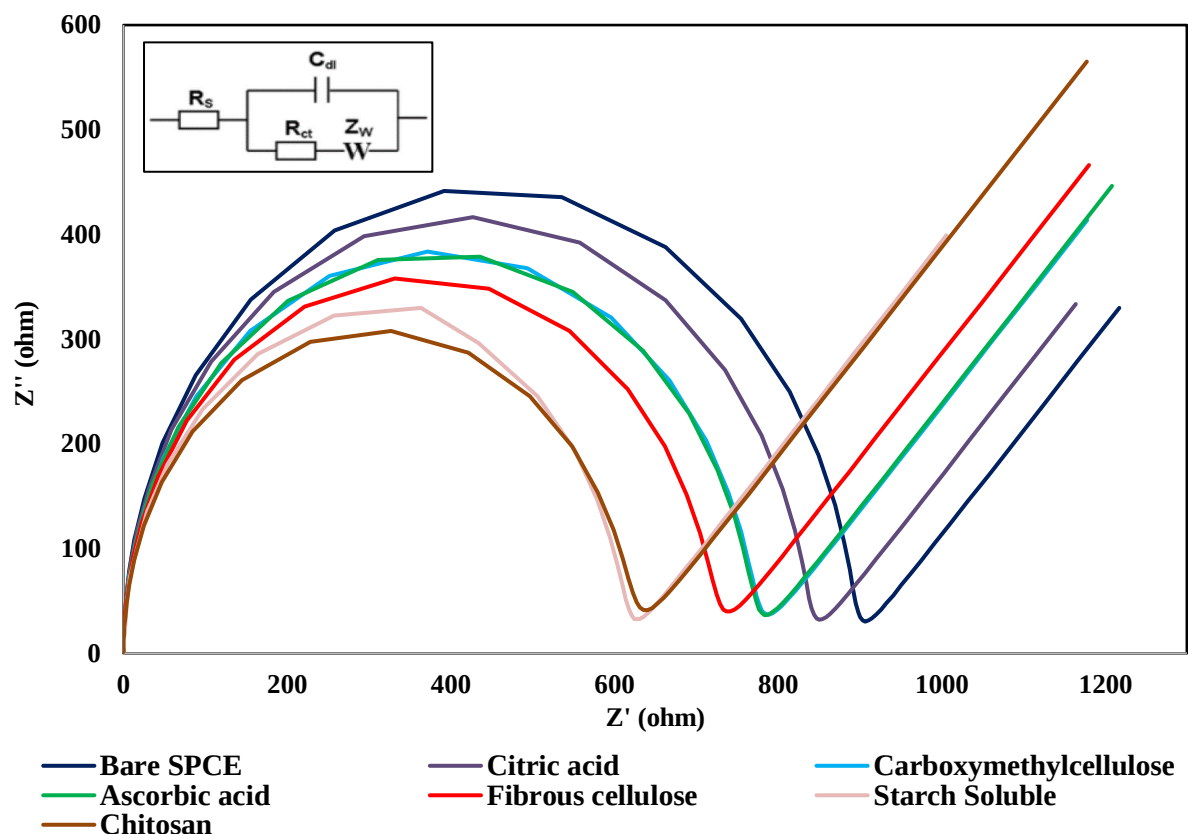


Figure 3.4: Nyquist plots of bare SPCE strip and CNPs from different types of precursor modified SPCE strip. The inset of shows the Randle Equivalent Circuit.

3.3.3 Relationship between Size of CNPs and its Electrical Conductivity

Carbon nanomaterials possess desirable electrochemical activity, electrical conductivity, a large total tuneable surface area and biocompatibility, which can greatly improve the efficiency of electrochemical immunosensors from different aspects and promising their genuine potential uses for future applications (Fang & Wang, 2013; Wang & Dai, 2015). As reported earlier in Section 3.3.1, different types of precursor may produce different size of CNPs and based on the scatter plot at Figure 3.5 between the measurement of electron-transfer resistance (R_{et}) from nyquist plot by EIS analysis versus different size of CNPs synthesized from chitosan, starch, fibrous cellulose, ascorbic acid, CMC and citric acid, it shows linear correlation with R^2 value of 0.8355

between the electron-transfer resistance (R_{et}) and particles size of CNPs. As we know R_{et} is inversely proportional to the rate of electron transfer on the electrode surface, thus, it may explain that the smaller the size of the CNPs, the larger the total surface area provided by the CNPs for transfer of electron on the electrode surface and the greater the electrical conductivity performance of the CNPs (Zhao et al., 2015)

Among all the different types of CNPs that synthesized from different types of precursor, the smallest size of chitosan CNPs performed superior findings on the higher electrical conductivity and faster electron transfer rates at the electrode surface (Zhao et al., 2015). Therefore, the chitosan CNPs was chosen for further investigate on their biosensing properties in Chapter 4 and Chapter 5 for immobilization process and undergoes surface functionalization in order to develop an electrochemical biosensing pave way for a reliable, rapid, accurate, simple, sensitive, selective and cost-effective detection of JEV (Muniandy et al., 2019).

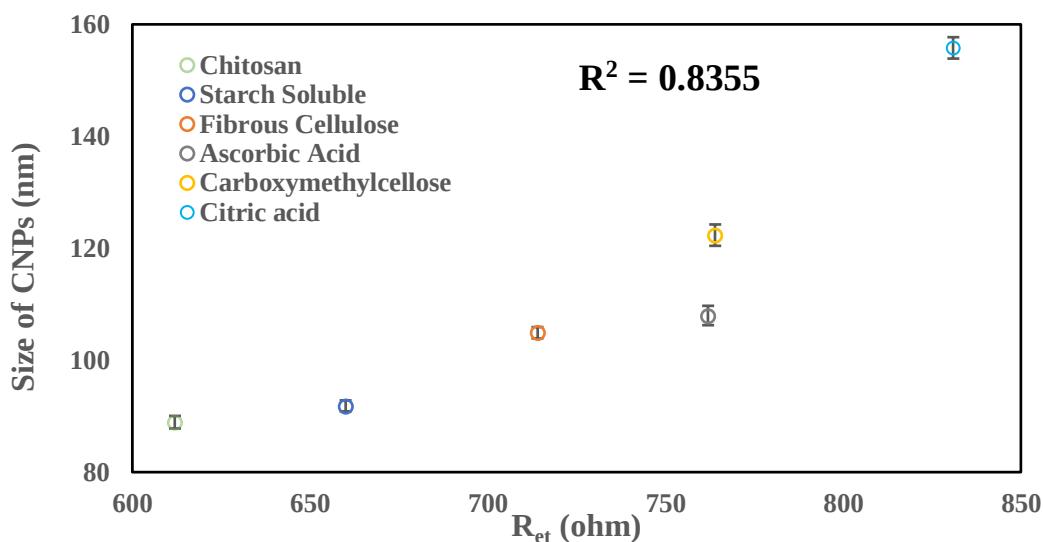


Figure 3.5: Scatter plot showed the relationship between the different size of CNPs from different types of precursor versus electron-transfer resistance (R_{et}). (Y error bar is calculated from standard deviation of particles size for 50 discrete and spherical NPs)

3.3.4 Optimization for the Temperature on Preparation of Chitosan CNPs

Remarkably, chitosan with the properties of non-toxic, natural carbon sources, biocompatible and biodegradable polysaccharide obtained from *N*-deacetylated derivative of chitin (a naturally abundant mucopolysaccharide) with average molecular weight ranging between 4 and 20 kDa (Liu et al., 2016) and containing high amount of amino (-NH₂) and hydroxyl (-OH) functional groups that in acidic pH may undergo protonation leading to its solubilization in water (Masotti et al., 2007). Chitosan-based nanoparticles would attract great interest from researchers from various countries due to their rapid process, simple, and accurate measurements.

CNPs with larger surface area is valued as a tightly adhering and biocompatible porous polymer providing secure fixation of the enzyme to the biosensor surface *via* physical entrapment in the pores (Gomes et al., 2017). However, adhesive film-forming properties and numerous oxygen and nitrogen residues that play a role in chemical modifications are the additional advantages of chitosan nanoparticles in electrochemical immunosensor (Solanki et al., 2011; Suginta et al., 2013; Abo Hamad et al., 2016).

Preparing CNPs *via* refluxing with acid becoming more popular recently in field research as a much shorter time is needed compared to the hydrothermal synthesis method, therefore the optimization of the reflux temperature until an optimum condition is achieved is crucial to produce a CNPs with high sensitivity electrochemical immunosensors. To enhance the performance of the CNPs, the different refluxing temperature of room temperature, 50 °C, 80 °C, 100 °C, 130 °C, 150 °C and 180 °C had been carried out for the preparation of CNPs and optimized. Results for the colour of CNPs solution, SEM micrograph, FTIR, XRD, CV and EIS are shown below at Figure 3.6 to Figure 3.10.

Figure 3.6 showed the colour of CNPs with different reflux temperatures. Chitosan nanoparticles turned black CNPs at 50 °C and above. However, to confirm and identify molecular components and structures, the FTIR spectra of all the samples for chitosan CNPs prepared by various temperatures were obtained and results showed in Figure 3.7. From the FTIR spectra, an obvious peak of amino groups (NH_2) at 1619cm^{-1} (Yang et al., 2012) was observed in the Fourier Transform Infra-red (FTIR) spectrum of CNPs as shown in Figure 3.7. This indicated that amino groups on chitosan nanoparticles have remained after the carbonization process. The amino groups on surfaces of CNPs were used to form amide bonds with carboxyl groups of the JEV antibody through a coupling reaction leading to immobilization of the antibody on the CNPs (Roy et al., 2011). However, based on the spectrum, CNPs synthesized using different reflux temperature do not show any significant difference in the absorption peak for CNPs synthesized *via* different temperature.

Size of the particles synthesized at the different reflux temperatures were analysed using Smile View Software and it is reported that the particles obtained from different reflux temperature may result in various particle sizes of CNPs as shown in Figure 3.8. The particle size ranging from ± 810 nm for particles synthesized at 50 °C to ± 89 nm for CNPs prepared at reflux temperature of 180 °C and details of the particle size synthesis at different reflux temperature were also reported at Figure 3.9.

With increased reflux temperature, the particle sizes become smaller and CNPs were able to disperse better in water, because of the high concentration of carboxylic groups on the surface. In addition, Figure 3.10 showed the XRD patterns for chitosan CNPs refluxed at 180 °C. It is seen that there are two humps at around $20^\circ - 25^\circ$ and $40^\circ - 45^\circ$. The characteristic peaks positioned at $2\theta = 20^\circ - 25^\circ$ indicate the amorphous and graphitic nature

of the materials, respectively while the second hump positioned at $2\theta = 40^\circ - 45^\circ$ indicate the presence of amorphous carbon (Shang et al., 2015; Dongol et al., 2015).

Combination all the data obtained from SEM, Colour, FTIR, and XRD, it can concretely confirm that CNPs has been formed. For chitosan CNPs, treatment at 180 °C and 30 min led to the highest conductivity, indicating that the tiny CNPs and carboxylic groups on the surface enabled better dispersion to achieve superior conductivity.

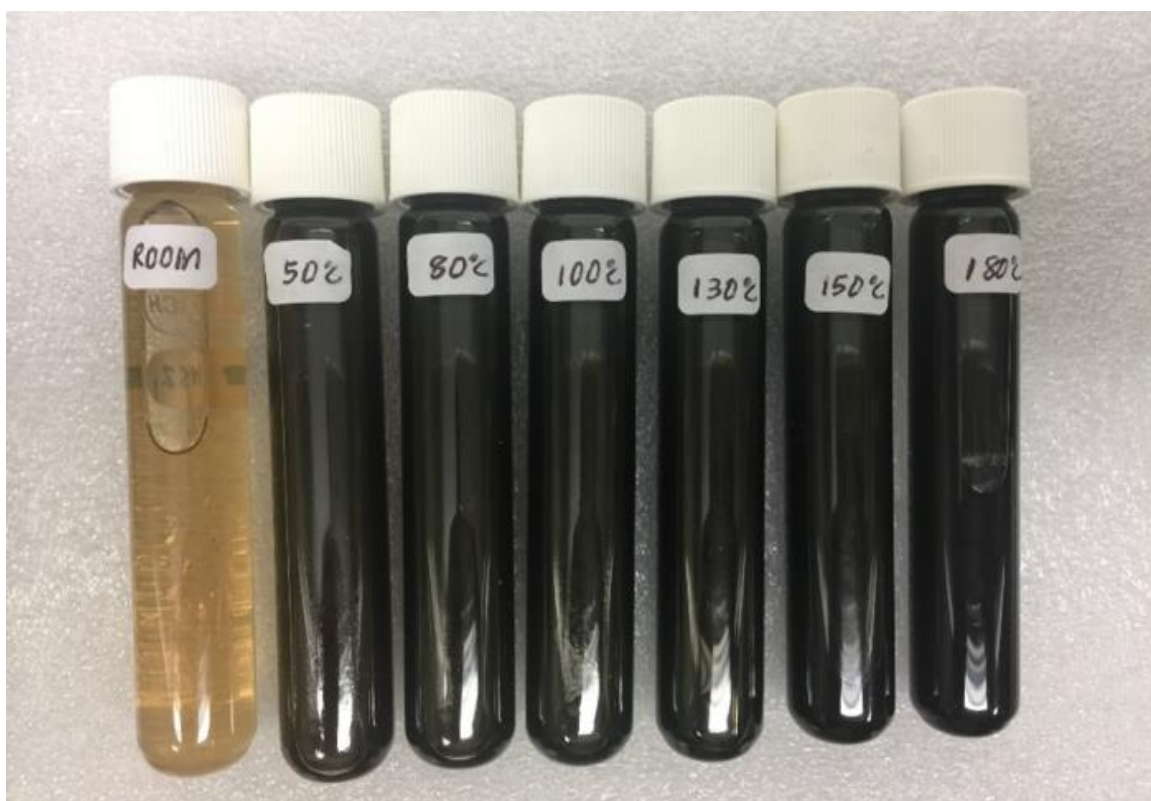


Figure 3.6: Colour of chitosan CNPs prepared through various reflux temperature.

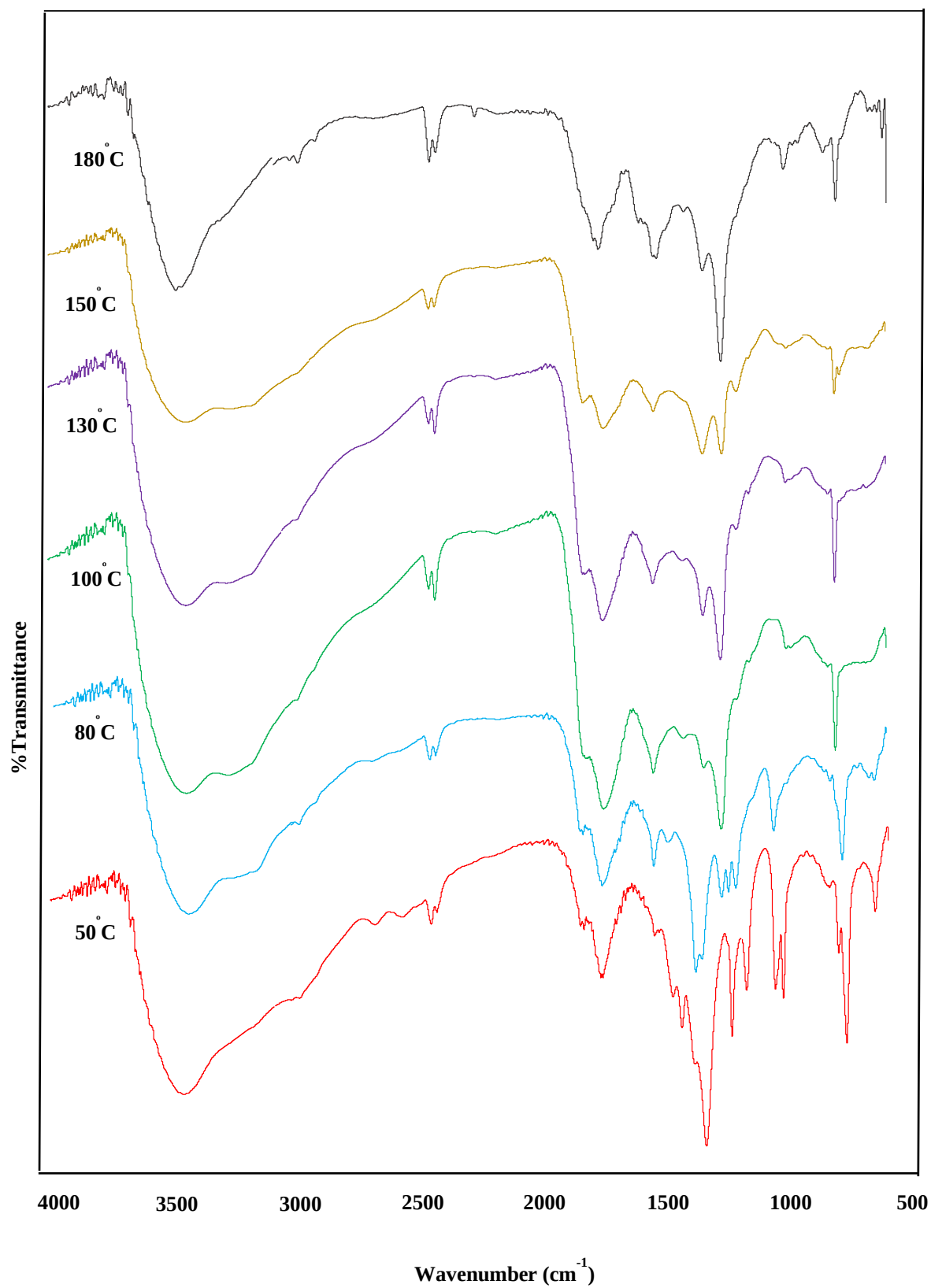
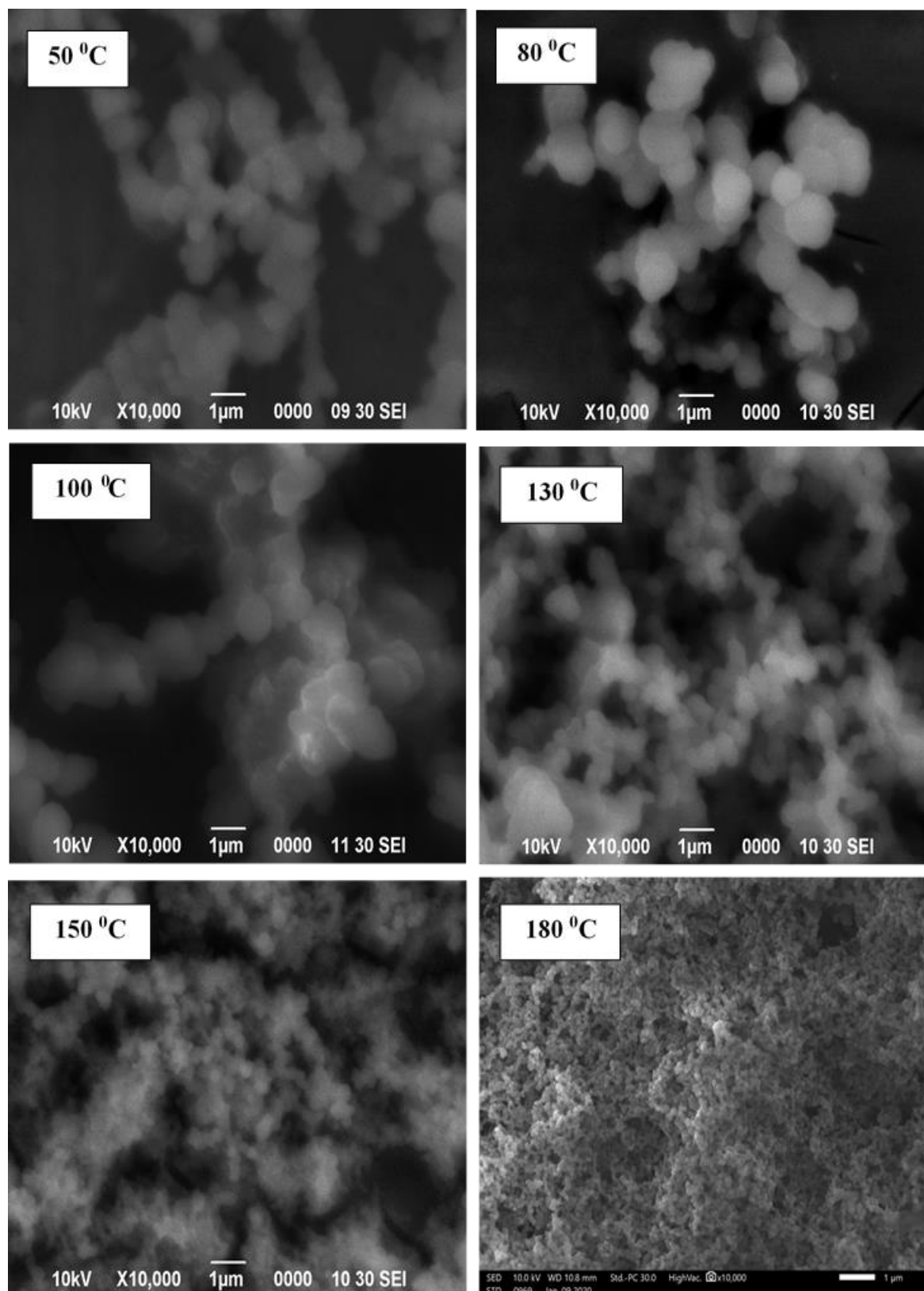


Figure 3.7: FTIR spectra for chitosan CNPs prepared through various reflux temperature.



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Figure 3.8: SEM micrograph of the particles prepared through various reflux temperature.

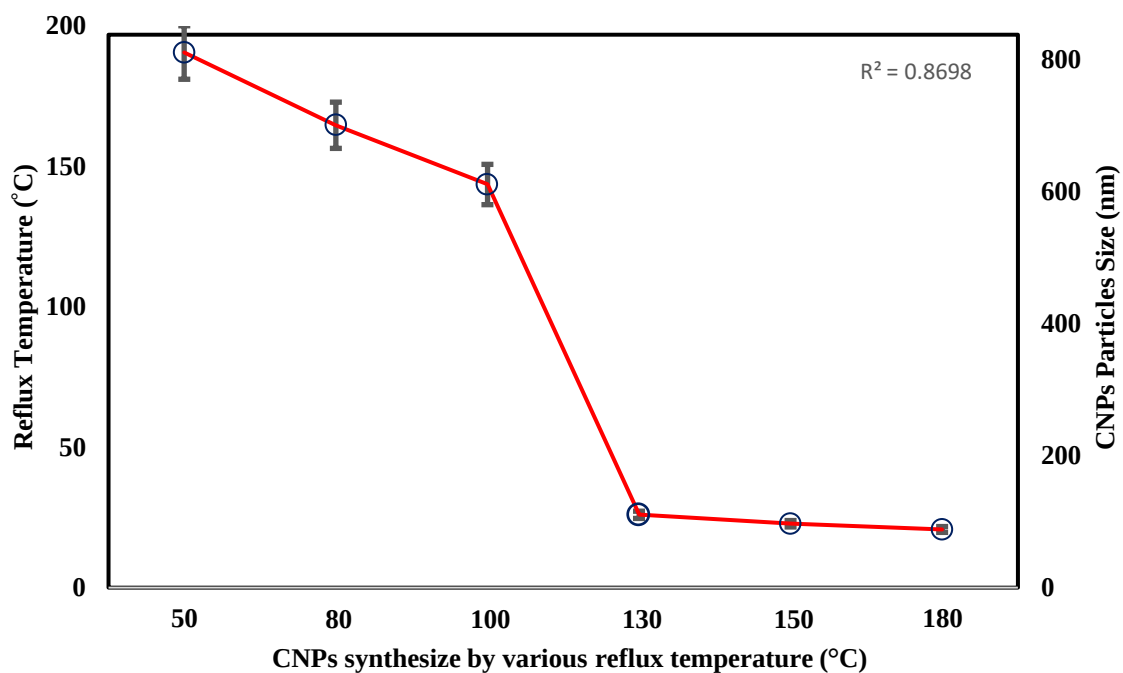


Figure 3.9: Scatter plot showed the relationship between the different size of particles from different types of precursor versus electron-transfer resistance (R_{et}). (Y error bar is calculated from standard deviation of particles size for 50 discrete and spherical NPs).

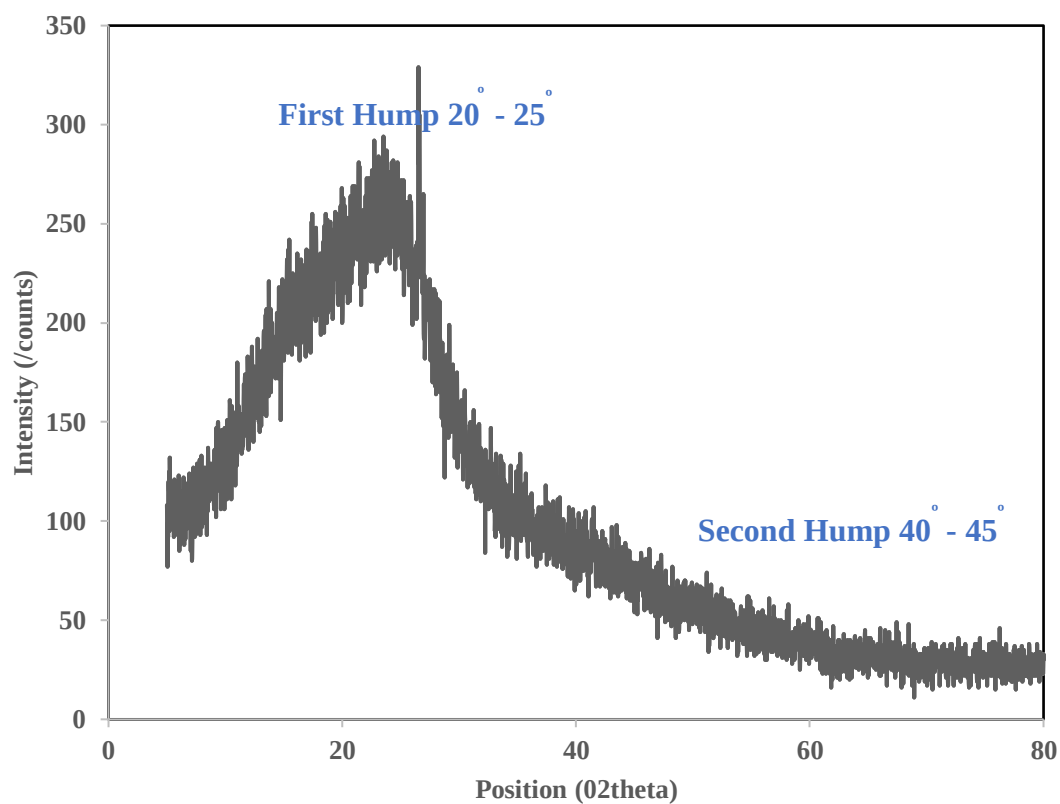


Figure 3.10: XRD pattern of chitosan CNPs reflux at 180 °C.

However, this finding can be proven by the electrochemical properties analysed by using cyclic voltammetry (CV) and Nyquist plots from electrochemical impedance spectroscopy (EIS) as shown in Figure 3.11 - Figure 3.12. It is interesting to report that CNPs synthesized at 180 °C exhibited the highest magnitude of both anodic and cathodic current showed on the voltammogram at Figure 3.11 and lowest electron-transfer resistance (R_{et}) as shown at Figure 3.12. Thus, based on the results from all the parameters tested, (i.e. the size of the particles with different reflux temperature and the electrical conductivity studies from CV measurement and electron-transfer resistance (R_{et}) from EIS studies, reflux temperature of 180 °C had attained for optimal sensing performance.

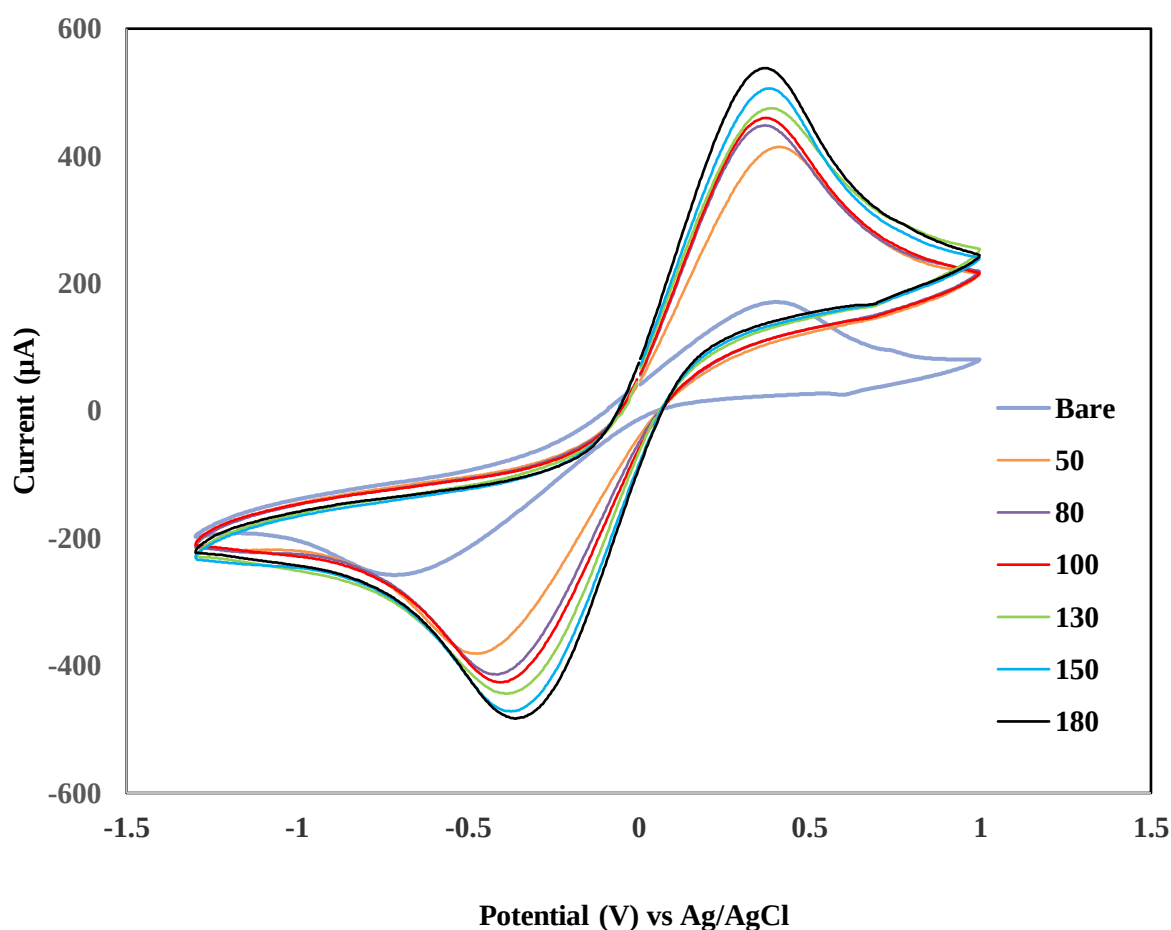


Figure 3.11: CV of bare SPCE strip and chitosan CNPs prepared at 50 °C, 80 °C, 100 °C, 130 °C, 150 °C and 180 °C.

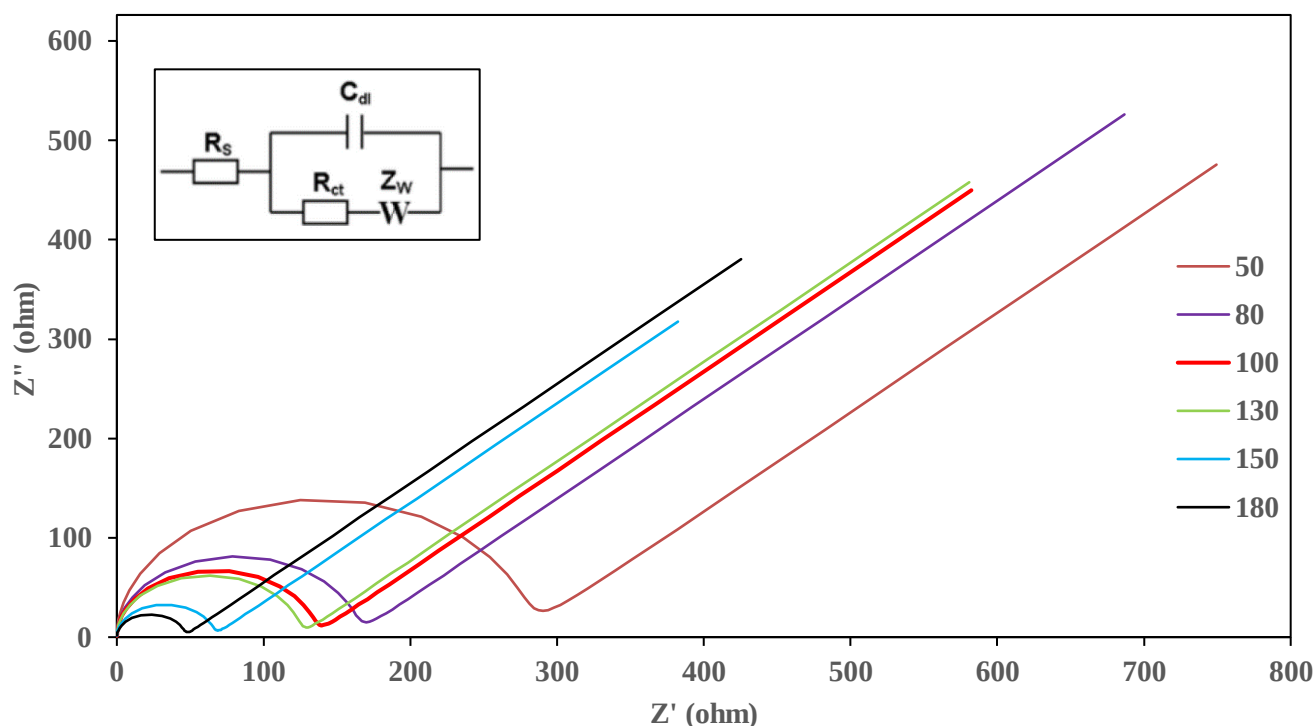


Figure 3.12: EIS of bare SPCE strip and chitosan CNPs prepared at 50 °C, 80 °C, 100 °C, 130 °C, 150 °C and 180 °C. Inset shows Randle Equivalent Circuit.

3.4 Conclusion

CNPs for different types of precursors (chitosan, starch, fibrous cellulose, carboxymethylcellulose, ascorbic acid and citric acid) can be prepared by refluxing with acid. From all the CNPs synthesized from various initiator, chitosan CNPs exhibited as the smallest size of the CNPs (± 89 nm) and the highest magnitude of current in the properties of electrical conductivity which mean the smaller the particles size of the CNPs that we synthesized, the higher the rates of electron transfer between the CNPs and the electrode surface and indirectly caused the electronic properties become greater.

Due to chitosan functional properties of nontoxic, natural carbon sources, biocompatible, biodegradable, simple and rapid preparation process, optimization studies on reflux temperature are also being carried out and findings showed reflux temperature of 180

°C is the optimum condition for the preparation of CNPs that can use to enhance the sensing performance of electrochemical biosensor.

During the nano-generation, CNPs have attracted numerous attentions as a substrate for electrochemical biosensor. Advances in this area are appearing frequently, with a multitude of significant breakthroughs taking place in the past two decades. The unique properties of CNPs have determined the rapid progress of CNPs-based electrochemical biosensors. As advanced materials, CNs possess desirable electrochemical activity, conductivity, a large but tuneable surface and biocompatibility, which can greatly improve the capabilities of electrochemical biosensors from different aspects. In relation to this, the successive chitosan CNPs synthesized in these studies will further investigate by undergoes immobilization or functionalisation that will discussed in the next chapter (i.e. Chapter 4 and Chapter 5). Therefore, signal acquisition and amplification can be conveniently accomplished in CNPs-based electrochemical biosensors, promising their genuine potential for future application.

CHAPTER 4

CARBON NANOPARTICLES (CNP_s) BASED ELECTROCHEMICAL BIOSENSOR STRIP FOR DETECTION OF JAPANESE ENCEPHALITIS VIRUS (JEV)

4.1 Introduction

Japanese encephalitis (JE) is an infectious disease caused by flavivirus viruses that affects human's brain membrane, and it is an endemic in China, India and Southeast Asia (Tran et al., 2012b; Bonaparte et al., 2014). Currently, the treatment options for Japanese Encephalitis virus (JEV) are limited and not often successful. Early diagnosis is therefore critical in controlling JE outbreak and preventing development of long-term serious symptoms in patients (Hombach et al., 2005; Tran et al., 2012a). The conventional diagnostic methods for JEV include Enzyme-linked Immunosorbent Assays (ELISA), Plaque Reduction Neutralisation Test, Reverse Transcription Polymerase Chain Reaction (RT-PCR), and virus isolation (Tran et al., 2012b; Tuan et al., 2013; Bonaparte et al., 2014). However, these methods require costly and bulky laboratory facilities, tedious diagnostic procedures, specially trained personnel, and long assay time.

Most JE cases happen in rural settings in Southern and Eastern Asian Countries, where diagnostic laboratory facilities are very limited (Coker et al., 2011). A portable diagnostic system that can provide point-of-care, rapid, sensitive detection of JEV is highly desirable for such settings (Jones et al., 2008). Electrochemical biosensors have received much attention as a reliable diagnostic tool for infectious diseases as their sensing performances are not affected by turbidity or absorbance of the sample (Bakker & Telting-Diaz, 2002). Besides, electrochemical biosensors offer the advantages of being highly sensitive, rapid in signal generation and readout, highly amenable for miniaturization,

inexpensive for virus detection, and requires relatively simple operational instrumentation (Pejcic et al., 2006).

To date, very few studies on electrochemical biosensors for JE diagnosis have been reported. Recently, an electrochemical biosensor was fabricated for the rapid detection of JE antigen by immobilized serum antibody on a silanized surface of an interdigitated sensor with a detection limit of $0.75 \mu\text{g mL}^{-1}$ within 20 minutes (Huy et al., 2011). In another study, gold-coated magnetite beads were used for the immobilization of JEV antibody and multiwall carbon nanotube (MW-CNT) was used to improve the signal as well as the conductivity of electrochemical biosensors. However, this method suffers from setbacks of requiring expensive precursor materials and electrode regeneration process prior to every single diagnosis (Li et al., 2011). Polyaniline nanowires modified interdigitated platinum (Pt) microelectrode electrochemical immunosensor with a detection limit of fewer than 10 ng mL^{-1} have also been reported. Nonetheless, the interdigitated platinum (Pt) microelectrode used was not disposable, thus suffer a setback of cross-contamination problems (Tuan et al., 2013).

Many studies have attempted to incorporate various types of nanoparticles such as silver nanoparticles, gold nanoparticles, graphene and carbon nanotubes, etc., into electrodes of biosensors in order to enhance the detection sensitivity and response time of biosensors (Huang et al., 2012a; Taleat et al., 2014; Tan et al., 2017). Nanoparticles afford very high surface area to volume ratio, which leads to a substantial increase in the loading of recognition elements on the electrodes and more efficient electrons transfer, and, in turn, enhances the sensitivity of the biosensors. However, these attempts were made on expensive precursor materials for the synthesis of nanoparticles and resulting in overall higher

production costs and therefore, low affordability for practical applications. Moreover, no long-term environmental effects have been investigated on the disposal of metal (e.g gold, silver) nanoparticles. On the other hand, CNPs have not been explored extensively for electrochemical biosensor fabrication. CNPs offer several advantages such as excellent electrochemical properties, inertness, low cost, low toxicity and good chemical stability (Sun et al., 2006; Lu et al., 2012; Yang et al., 2012). Herein, we report an electrochemical biosensor strip based on carbon nanoparticles modified SPCE for detection of Japanese Encephalitis Virus (JEV) with high sensitivity and selectivity. The clinical applicability of this electrochemical biosensor strip for the detection of JEV in human serum samples has been demonstrated.

4.2 Materials and Methods

4.2.1 Chemicals

All chemicals were of reagent grade and used without further purification. Sodium tripolyphosphate (TPP) of technical grade (85%), Bovine Serum Albumin (BSA), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (98%) (NHS) were supplied by Sigma-Aldrich (St. Louis, USA). Acetic acid was from HmbG Chemicals (Hamburg, Germany), chitosan powder with molecular weight of 100–300 kDa was purchased from Acros Organics (New Jersey, USA), Sulphuric acid of technical grade (95%) was purchased from J.T.Baker (Thailand), Phosphate buffer saline (PBS, pH 7) was obtained from Merck (Germany), Acetic acid glacial ($C_2H_4O_2$), Potassium ferricyanide ($K_3[Fe(CN)_6]$) and potassium ferrocyanide ($K_4[Fe(CN)_6]$) were obtained from Bendosen (Bendosen Laboratory Chemicals, Bendosen, Norway). Screen-printed carbon electrodes (SPCE) with a carbon-based working electrode, carbon-based counter electrode, and a silver/silver chloride (Ag/AgCl) reference electrode

were purchased from a local vendor Rapid Genesis Sdn Bhd. Ultra-pure water (18.2 MΩ.cm) was obtained from a water purifying system (ELGA Model: Ultra Genetic). JEV specific monoclonal antibody (Clone ID: MV12/1/C2–2/1), JEV antigen, JEV positive and JEV negative human serums (Dengue antigen) clinical samples were provided by the Institute of Health and Community Medicine (IHCM), Universiti Malaysia Sarawak (UNIMAS) and these biological products were stored at -20 °C.

4.2.2 Synthesis of Carbon nanoparticles (CNPs)

Successive chitosan CNPs synthesized in Chapter 3 is undergoes further investigation for immobilisation and functionalisation to enhance their sensitivity and selectivity properties. CNPs were prepared from preformed chitosan nanoparticles. The chitosan nanoparticles were prepared by crosslinking chitosan with sodium tripolyphosphate (Fan et al., 2012) *via* the ionic gelation method (Avadi et al., 2010). Initially, 0.3 g of chitosan powder were dissolved in 2% (v/v) 100 mL acetic acid glacial under stirring at room temperature to obtain 0.3% (w/v) chitosan solution. CNPs were prepared by adding sodium tripolyphosphate 1% (w/v) solution dropwise to chitosan solution under gentle magnetic stirring at room temperature. The chitosan nanoparticle suspension was centrifuged for 30 min at 13,500 rpm, and the supernatant obtained was heated with 8 mL of concentrated sulphuric acid *via* reflux at 180 °C, 30 minutes by using paraffin oil bath and after the black solution cooled down to room temperature, 40 mL of ultra-pure water is added to precipitate the black CNPs and the black CNPs were collected by the process of centrifugation (13,500 rpm).

4.2.3 Isolation of JEV

The Nakayama strain JEV was originally passage from suckling mouse brain

preparations and was subsequently maintained by passaging through mosquito cells, C6/36. The domain III region was cloned into a commercially available pET-SUMO vector (Invitrogen) and was expressed as a fusion protein with polyhistidine in *E.coli* cells, BL21-DE3 with isopropyl-B-D thiogalactopyranoside (IPTG) induction. The JEV was purified using a Ni^{2+} chelating chromatographic column under denaturing condition and then dialysed into phosphate buffer saline (Liddell, 2001b; Cardoso et al., 2002; Sum et al., 2015).

4.2.4 Activation of the JEV antibody and immobilizing on the surface of CNPs

A monoclonal antibody (Clone ID: MV12/1/C2-2/1), raised in a mouse-specific to produce immune response to JEV, and originally provided by Venture Technology, Penang, Malaysia (Cardoso et al., 2002; Sum et al., 2015), was treated with ethyl(dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) for activation. 200 μL each of ethyl(dimethylaminopropyl) carbodiimide (EDC, 0.2 M) and N-Hydroxysuccinimide (NHS, 0.05 M) was added to 200 μL of JEV antibody (10.0 M) to activate its carboxyl groups by allowing them to react for 1 hour at 4 °C. The activated JEV antibody was then added onto the surfaces of CNPs at room temperature and then incubated for 24 hours to immobilize the JEV antibody on CNPs surfaces by allowing the formation of amide bonds between amino groups on CNPs surface and activated carboxyl groups on the JEV antibody.

4.2.5 Instrumentation

The morphology of samples was observed using a scanning electron microscope (SEM) (JEOL JSM-6390 LA). Samples were coated with a thin layer of platinum prior to SEM analysis. The mean diameter (particles size) of the chitosan CNPs obtained were determined using the Smile View Software by measuring the diameter of at least 50 discrete

and spherical nanoparticles as observed in the SEM micrograph. FTIR spectra of samples were obtained from KBr sample pellets within the range of 400–4000 cm^{-1} on a FTIR spectrometer (Thermo Scientific, Nicolet iS10). Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were performed on a Potentiostat (Princeton Applied Research, PARSTAT 2263, using an electrolyte containing 15.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl. For CV characterization, the potential was cycled between -1.3 V and $+1.0$ V (vs Ag/AgCl) at a scan rate of 50.0 mVs^{-1} and the EIS measurements were recorded within the frequency range of 0.01 Hz to 100.0 kHz.

4.2.6 Preparation of CNPs-SPCE Electrochemical Biosensor Strip

10 μL of JEV antibody immobilised CNPs was dropped coated onto the working electrode of the SPCE strip and incubated for 48 hours at 37°C via physically binding mechanism and then 2% (w/v) bovine serum albumin (BSA) in PBS was then added onto the surface of the working electrode to block unsaturated and non-specific binding sites. The electrode was rinsed with PBS to remove excess BSA.

4.2.7 Detection of JEV Antigens

For detection of JEV antigens, 10 μL of JEV of various concentrations ranging from 1 to 20.0 ngmL^{-1} was dropped onto the CNPs modified SPCE electrochemical biosensor strip, incubated at 37°C for 20 min, rinsed and air-dried. The interaction of JEV with antibody immobilized on the surface of CNPs modified SPCE electrochemical biosensor strip working electrode was studied using EIS. The relative resistance was calculated based on Equation 4.1 below (Rezaei et al., 2011).

$$\text{Relative resistance} = \frac{Ret(i) - Ret(o)}{Ret(o)} \quad \text{Equation 4.1}$$

Ret (*i*) is the value of electron transfer resistance after the binding of JEV-to-JEV antibody and Ret (*o*) is the value of electron transfer resistance after the immobilization of JEV antibody onto CNPs modified SPCE electrochemical biosensor strip working electrode surface and block with BSA. Figures 4.1 illustrated the schematic diagram of preparation processes employed on the SPCE strip for fabricating electrochemical biosensor strip for the detection of JEV.

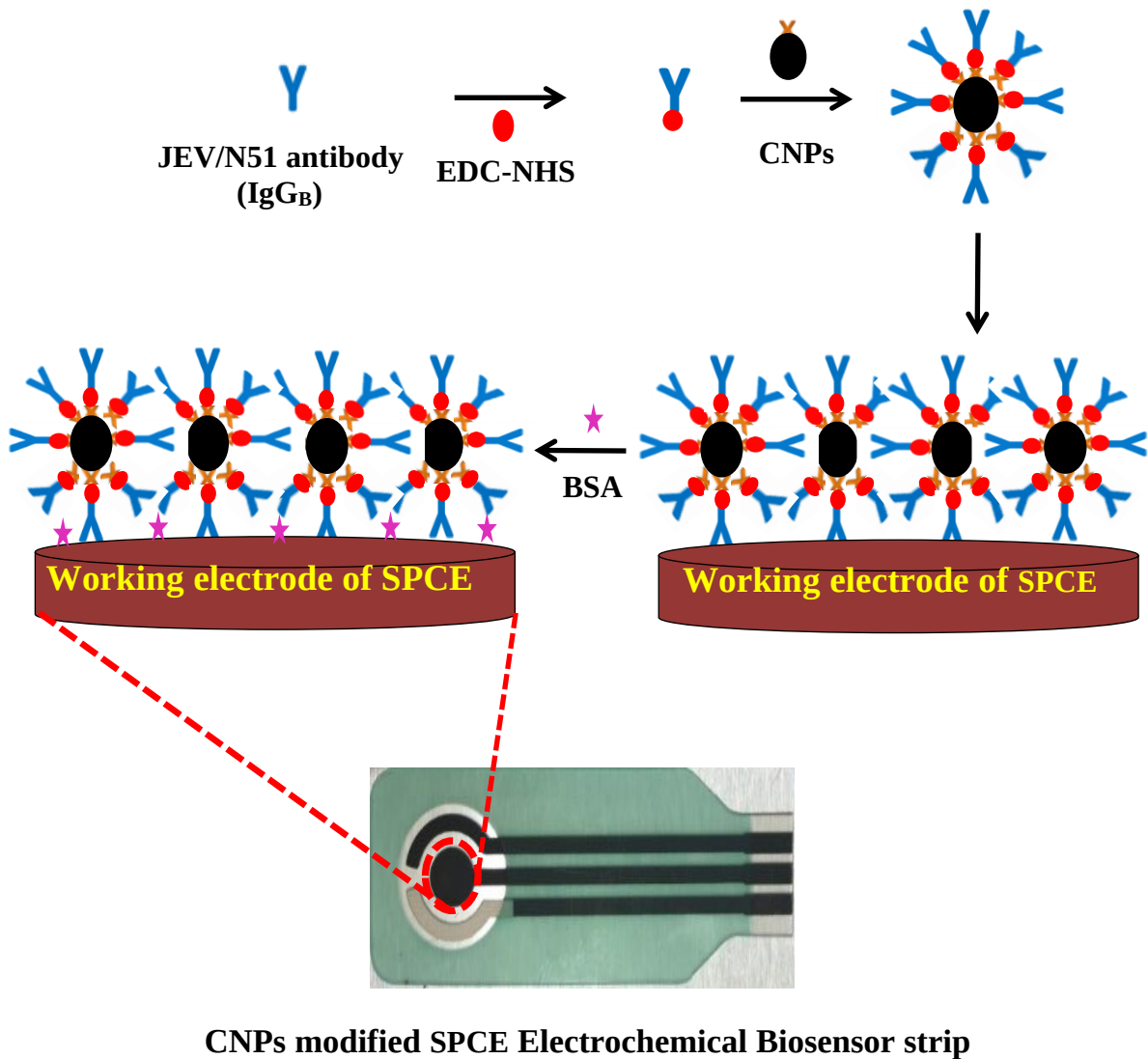


Figure 4.1: Schematic diagram of preparation processes employed on the SPCE strip for fabricating electrochemical biosensor for the detection of JEV (Lai et al., 2017).

4.3 Results and Discussion

4.3.1 Morphology of Chitosan Nanoparticles and Carbon Nanoparticles (CNPs)

Scanning electron microscopy micrographs (SEM) of chitosan nanoparticles and carbon nanoparticles (CNPs) used for immobilizing antibodies on the SPCE strip were shown in Figure 4.2 (A), (B) and (C), respectively. The mean particle size of the CNPs was around 89 nm. CNPs provide a large specific surface area for JEV antibody immobilization while enhancing the efficiency of electron transfer. Moreover, the amino group on the surface of CNPs can be used for attachment of the antibody.

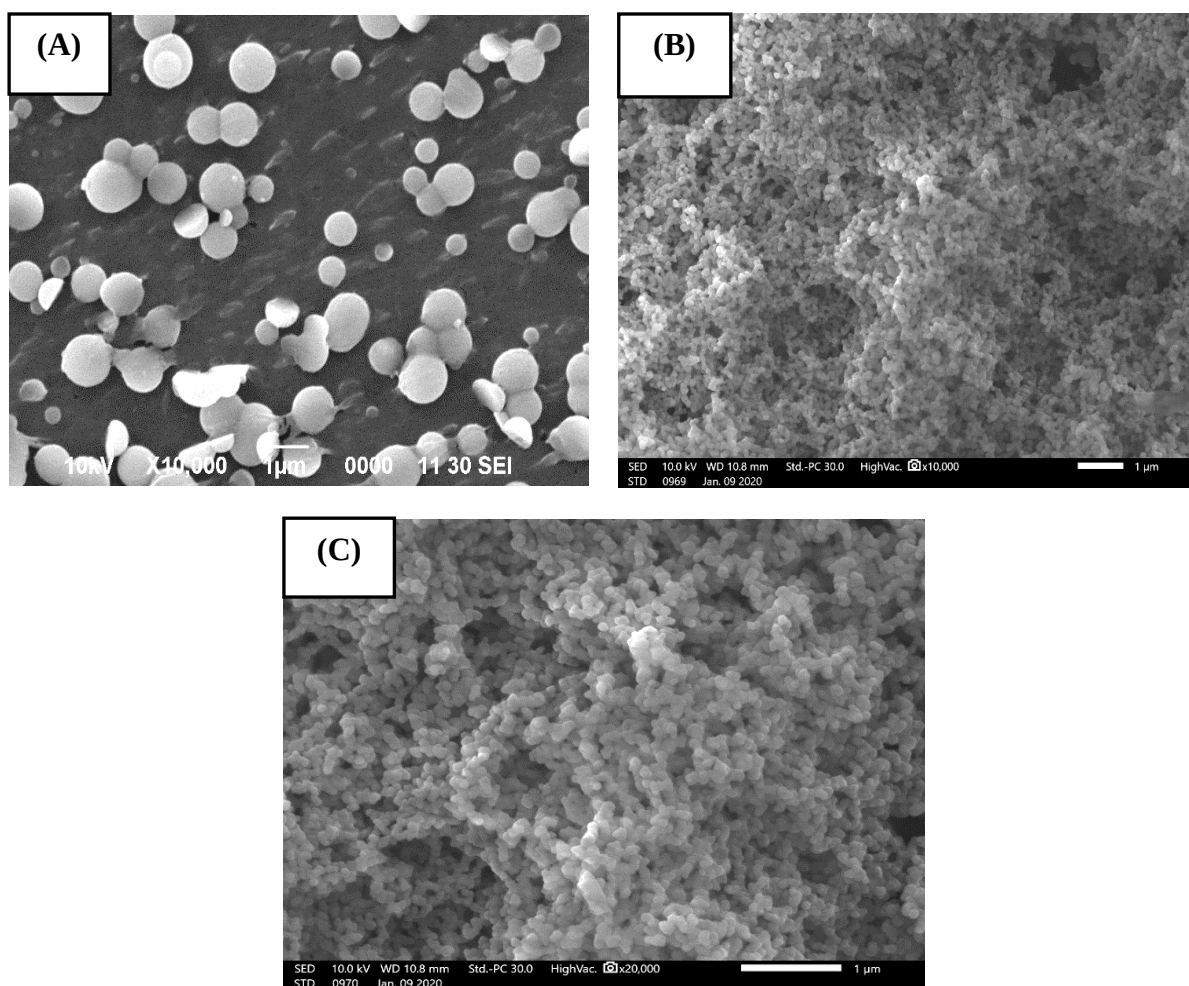


Figure 4.2: Scanning electron micrographs of (A) chitosan nanoparticles, (B) carbon nanoparticles (CNPs) in x10000 and (C) carbon nanoparticles (CNPs) in x20000 used for the immobilization of JEV antibody.

4.3.2 Characterization of Immobilized JEV Antibody onto CNPs

A distinctive peak of amino groups (N-H bending) at 1619.23 cm^{-1} (Yang et al., 2012) and N-H stretching peak at 3401.44 cm^{-1} was observed in the FTIR spectrum of CNPs as shown in Figure 4.3 (A). This indicated that amino groups on chitosan nanoparticles have remained after the carbonization process (Alaneed et al., 2021). However, theoretically, the N-H stretching peak at 3401.44 cm^{-1} should be a sharp peak, but both spectra in Figure 4.3 (A) and Figure 4.3 (B) showed broad peaks and this could be the overlapping in between the N-H stretching band, O-H stretching band and C-H stretching band (Alaneed et al., 2021).

The amino groups on surfaces of CNPs were used to form amide bonds with carboxyl groups of the JEV antibody through a coupling reaction leading to immobilization of the antibody on the CNPs (Roy et al., 2011). The activated carboxyl groups of the JEV antibody underwent a coupling reaction after being dropped on the surface of CNPs at room temperature. The presence of a characteristic carbonyl (C=O) stretching of amide bonds absorption peak at around 1631 cm^{-1} (Sharma et al., 2010) in the FTIR spectrum of the JEV antibody immobilized on CNPs confirmed the formation of amide bonds between amino groups of CNPs and carboxylic groups of JEV antibody Figure 4.3 (B).

In addition, both spectra of Figure 4.3 (A) and Figure 4.3 (B) showed the peak for P=O presence at around 1119.81 cm^{-1} and this could be happened from the addition of tripolyphosphate compound during the synthesis of chitosan nanoparticles.

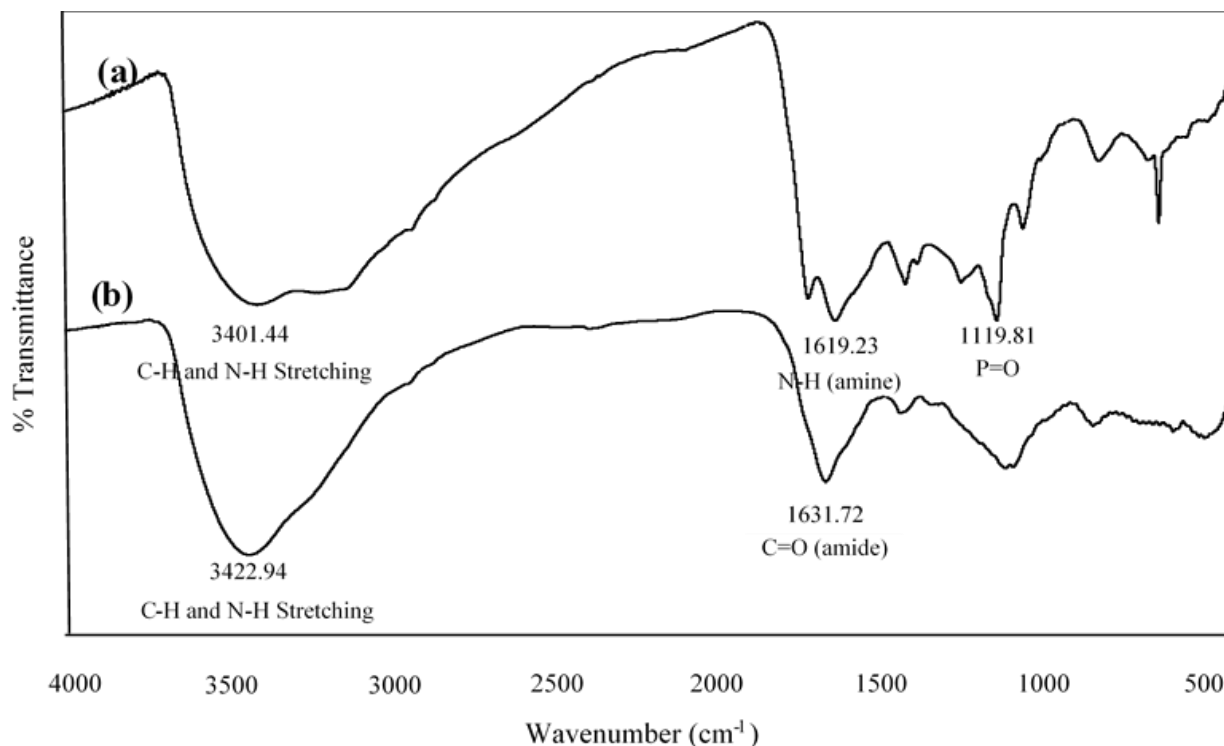


Figure 4.3: FTIR spectra of (A) carbon nanoparticles (CNPs), and (B) JEV antibody immobilized onto carbon nanoparticles.

4.3.3 Electrochemical Characterization of the Immunosensor

4.3.3.1 Cyclic Voltammetry (CV)

The JEV antibody immobilized CNPs modified SPCE electrochemical biosensor strip was characterized by CV in the presence of 15 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) as redox probes and 0.1 M KCl at pH ~7.4. CV in Figure 4.4 showed that CNPs modified SPCE electrochemical biosensor strip (curve ii) exhibited about 9.2% higher cathodic current density than unmodified SPCE strip (curve i). The presence of CNPs led to an increase in the conductivity and effective electroactive surface area of SPCE. The addition of JEV antibody immobilized CNPs onto SPCE strip resulted in an observable decrease of cathodic current (curve iii). The magnitude of cathodic current was further decreased upon the addition of BSA (curve iv) due to blocking of non-specific sites present on the working electrode that hindered electrons transfer. In the presence of JEV, the cathodic current was

observed to drop further (curve v). The presence of JEV was detected based on the highly specific recognition between JEV and the antibody immobilized on the CNPs. The formation of antigen-antibody complexes insulated the electrode by hindering the redox reaction of $K_3Fe(CN)_6/K_4Fe(CN)_6$ and diffusion towards the electrode surface which led to the observed decrease in cathodic current (curve v).

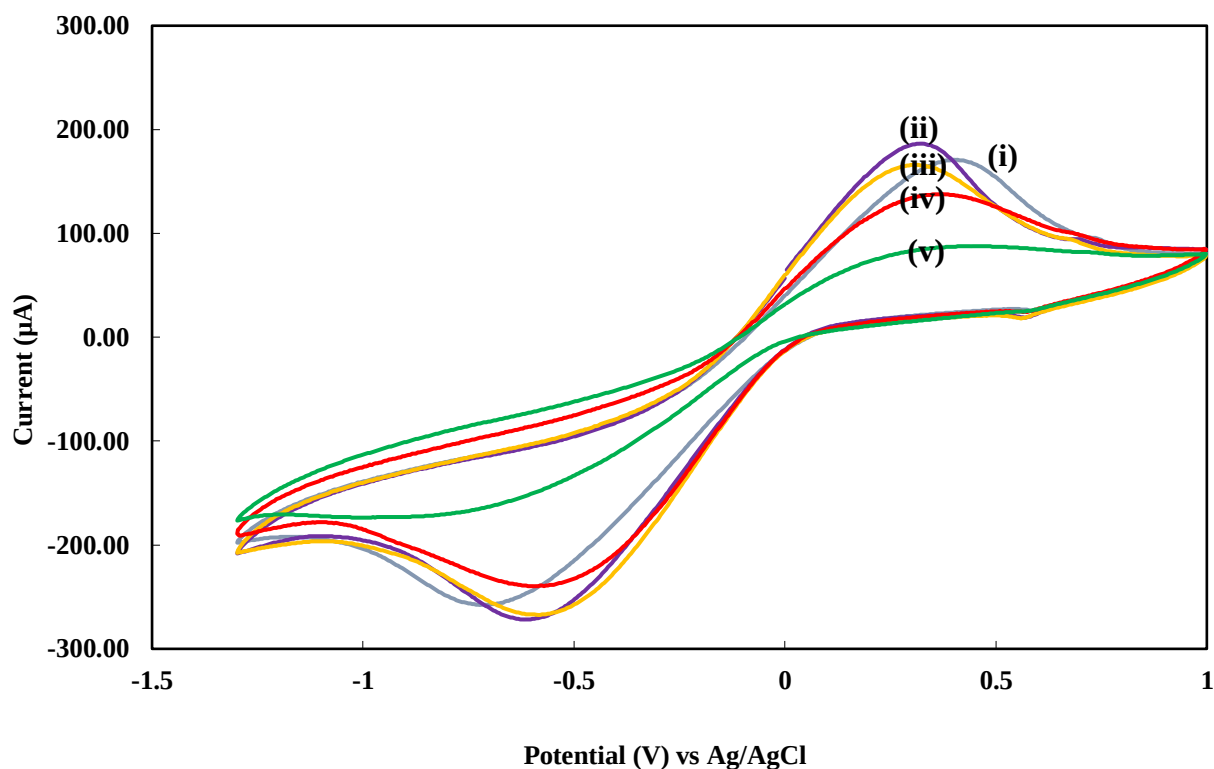


Figure 4.4: Cyclic voltammograms (CV) of (i) bare SPCE strip; (ii) CNPs modified SPCE strip; (iii) JEV antibody immobilized CNPs on SPCE strip; (iv) BSA blocked/JEV antibody immobilized CNPs on SPCE strip and (v) BSA blocked/JEV antibody immobilized CNPs on SPCE strip in the presence of JEV.

4.3.3.2 Electrochemical Impedance Spectroscopy (EIS)

Electrochemical impedance spectroscopy (EIS) measurements were carried out in the presence of 15.0 mM of $K_3Fe(CN)_6/K_4Fe(CN)_6$ (pH ~7.4) containing 0.1 M KCl in order to further investigate the effects of electron transfer on the electrode surface. All EIS data were simulated by the Randle equivalent circuit as shown in the inset of Figure 4.5. As observed in Figure 4.5, the Nyquist plot of unmodified SPCE electrochemical biosensor strip

displayed a larger semicircle of larger diameter (curve i) than that of CNPs modified SPCE electrochemical biosensor strip (curve ii). Unmodified SPCE had exhibited a higher electron transfer resistance ($R_{et} = 764 \text{ ohm}$) as compared with CNPs modified SPCE ($R_{et} = 660 \text{ ohm}$) due to faster electron transfer rates at the electrode surface. The immobilisation of the JEV antibody onto the surface of CNPs had resulted in an increase of R_{et} (curve iii) due to the presence of the hydrophobic layer of antibody which hindered interfacial electron transfer. As expected, R_{et} was observed to increase after the addition of BSA to block non-specific sites (curve iv). In the presence of JEV, the R_{et} was observed to further increase (curve v) which was attributed to the formation of an antigen-antibody non-conductive layer which blocked electron transfer at the electrode surface.

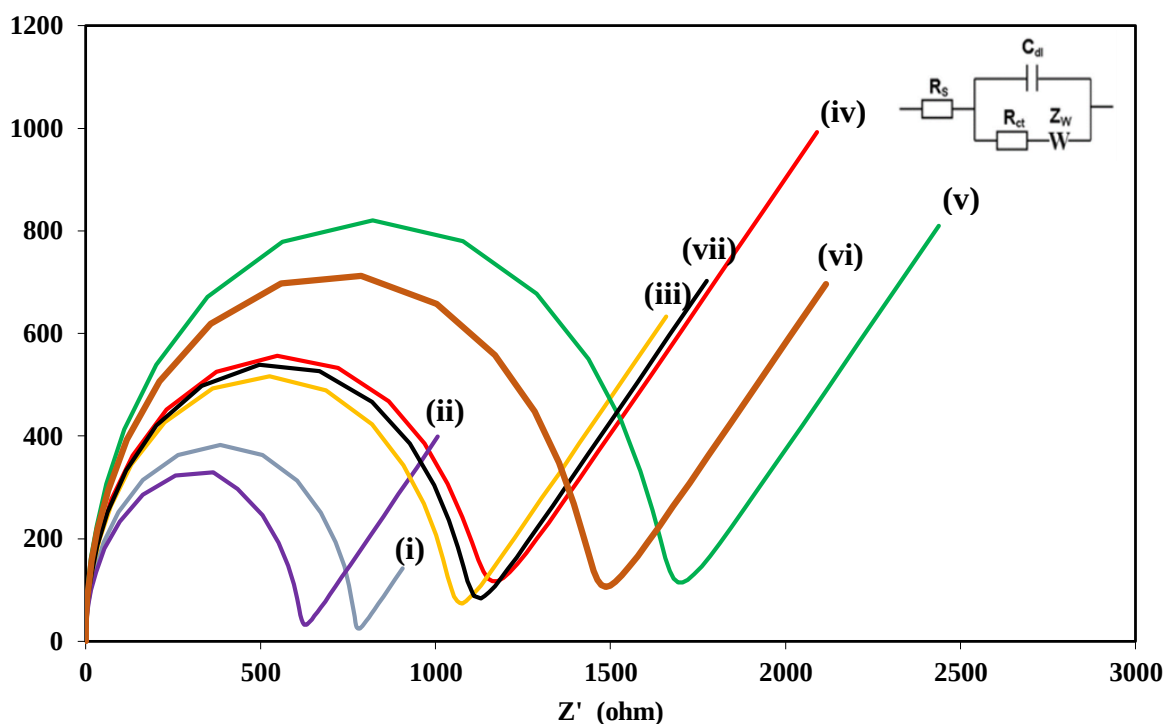


Figure 4.5: Nyquist plots of (i) bare SPCE strip; (ii) CNPs modified SPCE strip; (iii) JEV antibody immobilized CNPs on SPCE strip; (iv) BSA blocked/JEV antibody immobilized CNPs on SPCE strip; (v) BSA blocked/JEV antibody immobilized CNPs on SPCE strip in the presence of JEV; (vi) JEV positive human serum and (vii) Dengue antigen. Inset shows the Randle Equivalent circuit.

4.3.4 Specificity, Selectivity, and Capability

To demonstrate clinical applicability and selectivity of the CNPs-SPCE electrochemical biosensor strip, both JEV positive human serum clinical samples and Dengue antigen were used as the analytes and evaluated by using EIS. As observed in Figure 4.6, in the presence of sample containing JEV positive human serum (curve vi), the R_{et} (1421 ohm) was observed to be substantially higher as compared to the R_{et} (1099 ohm) of BSA blocked/JEV antibody immobilized CNPs on SPCE strips (curve iv). These results indicated that the CNPs-SPCE electrochemical biosensor strip was capable of JEV detection in human serums. The selectivity of this biosensor strip was proven as in the presence of Dengue antigen, the R_{et} (1073 ohm) of (curve vii) was observed to have no significant changes as compared with R_{et} (1099 ohm) of BSA blocked/JEV antibody immobilized CNPs on SPCE strips (curve iv). This is because the Dengue antigen was unable to form an antigen-antibody non-conductive layer with the antibody of JEV.

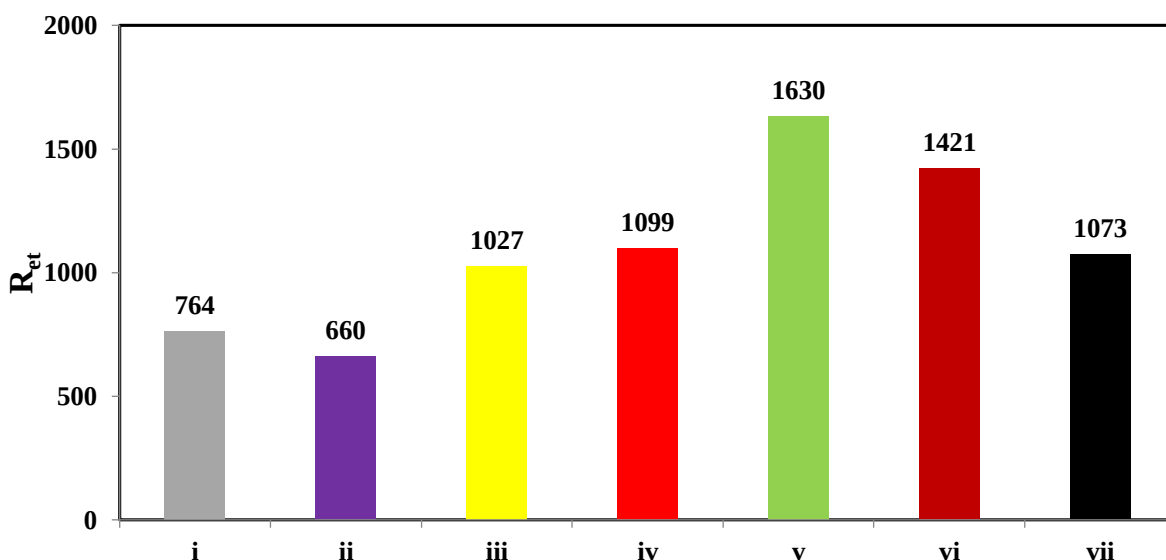


Figure 4.6: Bar chart of (i) bare SPCE strip; (ii) CNPs modified SPCE strip; (iii) JEV antibody immobilized CNPs on SPCE strip; (iv) BSA blocked/JEV antibody immobilized CNPs on SPCE strip; (v) BSA blocked/JEV antibody immobilized CNPs on SPCE strip in the presence of JEV; (vi) JEV positive human serum and (vii) Dengue antigen.

4.3.5 The LOD of Japanese encephalitis virus (JEV) based on EIS

The EIS spectra of CNPs-SPCE biosensor strip incubated with various concentrations of JEV were displayed in Figure 4.7 (A). The diameters of semicircles were observed to increase as the concentration of JEV was increased. Figure 4.7 (B) shows the calibration plots of relative resistance calculated by using Equation 4.1 versus concentrations of JEV ranging from 1 to 20 ngmL⁻¹, the relative resistance direct proportional to JEV concentration with R² value of 0.913 which mean the higher the concentration of JEV, the higher the relative resistance and the lower the R_{et} value from Nyquist plots. The increase in R_{et} could be attributed to the formation of more JEV antigen-antibody complexes at higher JEV concentration which will then inhibit the interfacial electron transfer on electrode. A linear correlation equation between relative resistance and JEV concentration (ngmL⁻¹) were obtained (Figure 4.7 (B)), as shown in Equation 4.2.

$$\text{Relative resistance} = 0.024 \text{ Conc JEV} + 0.186 \quad \text{Equation 4.2}$$

Concentration JEV is the concentration of JEV in ngmL⁻¹. The value of the regression coefficient (R²) and standard deviation (σ) were 0.91 and 0.19, respectively. The calibration plot of Relative Resistance versus concentrations of JEV ranging from 1 to 20 ngmL⁻¹ was used to determine the LOD for the biosensor strip. The CNPs-SPCE biosensor strip showed a very low detection limit of 0.36 ngmL⁻¹ for JEV. From equation 4.2, the detection sensitivity was 0.024 ngmL⁻¹. This detection limit is substantially lower than the previously reported detection limit of 10 ngmL⁻¹ for an interdigitated sensor (Tran et al., 2012b), and a polyaniline nanowire-based electrochemical immunosensor at below 10 ngmL⁻¹ (Tuan et al., 2013). The CNPs-SPCE biosensor reported herein exhibited equivalent to more than an order of magnitude higher in sensitivity for JEV detection.

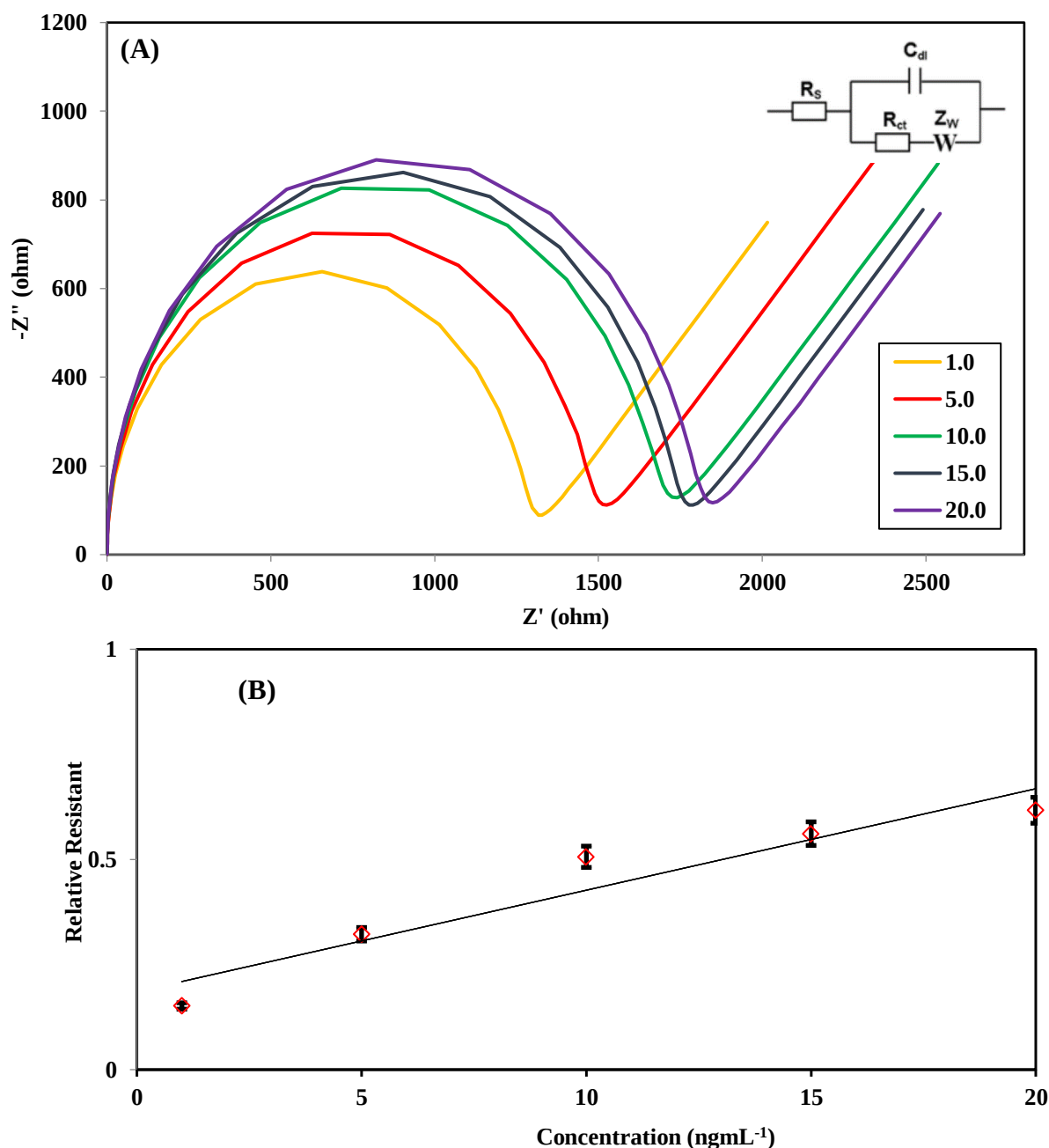
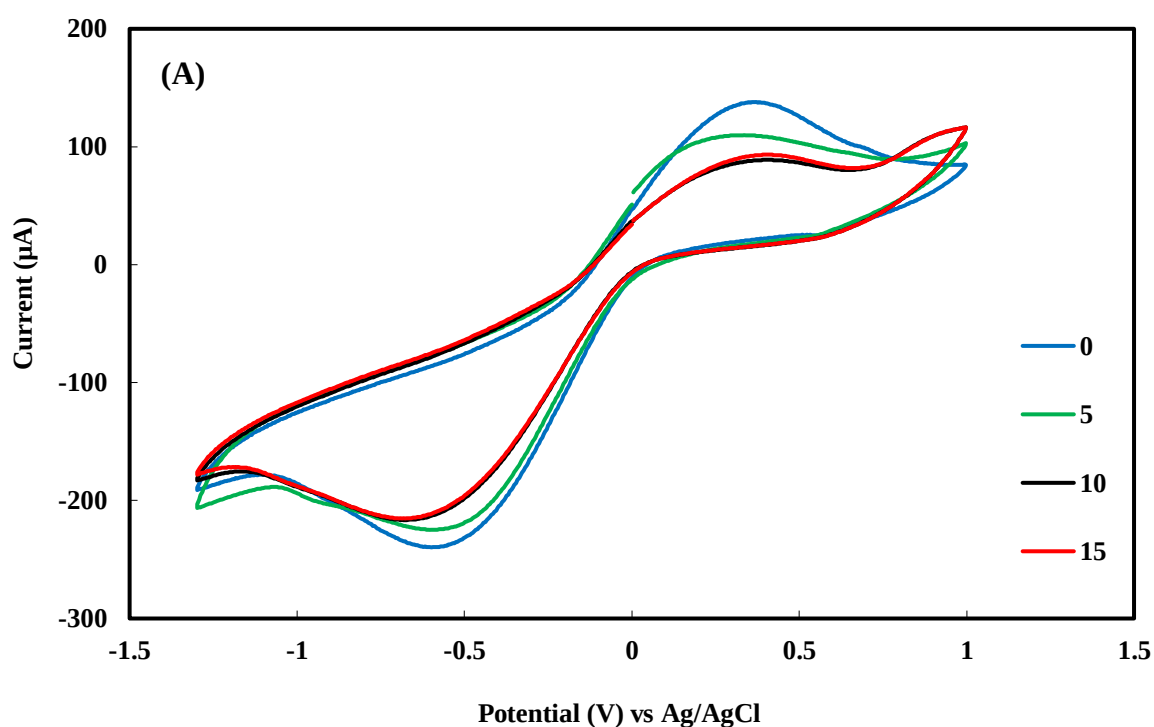


Figure 4.7: (A) Nyquist plots of JEV antibody immobilized CNPs on SPCE strip with different JEV concentrations (ng/mL), (B) show calibration plots of Relative resistance versus concentrations of JEV ranging from 1 to 20 ngmL^{-1} and Inset of Figure 4.7 (A) show the Randle Equivalent Circuit. (Error bars of Figure 4.7 (B) were calculated from the mean, $s/n = 3$).

4.3.6 Response Time of the Immunosensor

Cyclic voltammograms of CNPs modified SPCE electrochemical biosensor strip as a function of response time were shown in Figure 4.8 (A) and the plot of current (μA) versus

response time (min) were shown in Figure 4.8 (B). In the Figure 4.8 (B), the graph showed that the current is inversely proportional to the time for the first 10 minutes and become stable and constant after 10 minutes. This is because at the first 10 minutes, the formation of antigen-antibody complexes insulated the electrode by hindering the redox reaction of $K_3Fe(CN)_6/K_4Fe(CN)_6$ and diffusion towards the electrode surface which led to the decrease in current. However, the CNPs modified SPCE electrochemical biosensor strip was observed to achieve stable cathodic current in 10 minutes. This result demonstrated that the proposed biosensor strip exhibited rapid responses and results of the analysis were obtainable within 10 minutes. This observed response time is considerably faster than that reported for interdigitated biosensor at 20 minutes (Huy et al., 2011), and other JEV diagnosis methods which require diagnosis times ranging from hours to days (Taleat et al., 2014). The substantially enhanced sensitivity and faster response time could be attributed to the use of CNPs which enabled a higher loading of JEV antibody as well as the increased overall electrical conductivity of the SPCE biosensor strip.



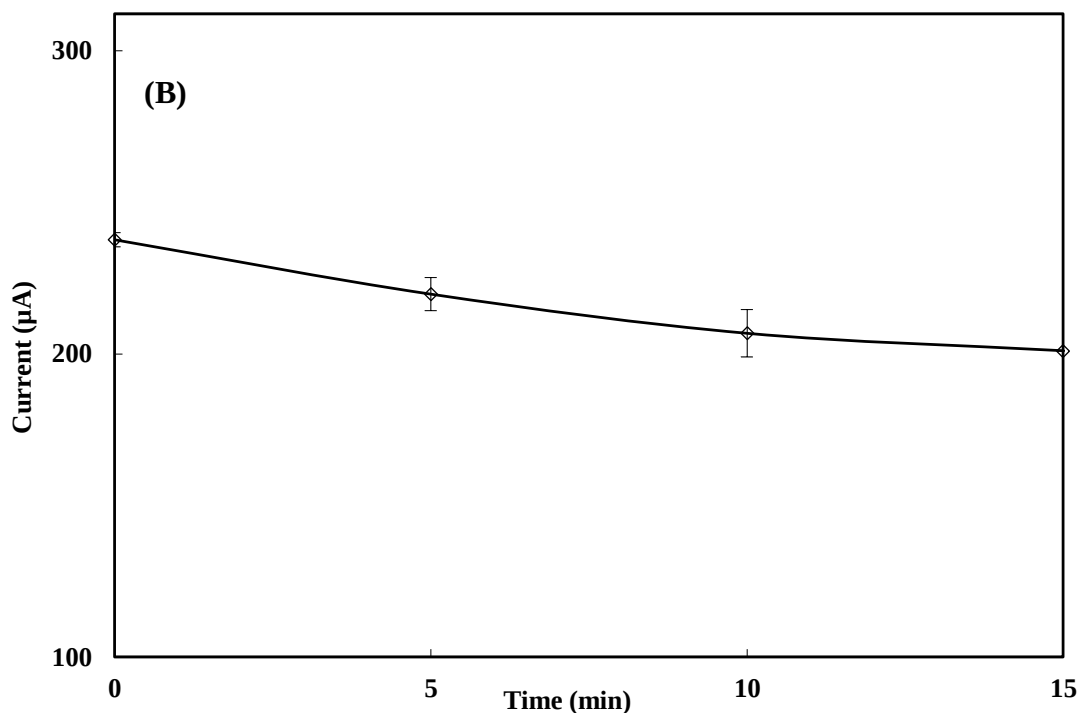


Figure 4.8: (A) Cyclic voltammograms of JEV antibody immobilized CNPs on SPCE biosensor strip as a function of response time: 0 min; 5 min; 10 min, and 15 min and (B) shows the plot of current (μA) versus response time (min).

4.4 Conclusion

In this work, the superior performance of chitosan CNPs prepared in Chapter 3 successfully immobilised with JEV antibody through the formation of amide bond between amine group for chitosan CNPs and carboxyl group of JEV antibody and a disposable CNPs modified SPCE electrochemical biosensor strip was successfully fabricated for the rapid and sensitive detection of JEV.

This biosensor strip can achieve a stable response signal within 10 minutes and has shown a limit of detection of 0.36 ngmL^{-1} and detection sensitivity were 0.024 ngmL^{-1} for JEV. The disposable nature of this biosensor strip will prevent cross-contamination from previous samples in clinical diagnosis.

However, the capability of the potential clinical application of this biosensor strip for detecting of JEV in human serum samples has been demonstrated and results indicated that the CNPs-SPCE electrochemical biosensor strip was capable for JEV detection in human serums and the selectivity of this biosensor strip also performed in this study by using Dengue antigen. It was observed to have no significant changes in R_{et} value as compared with BSA blocked/JEV antibody immobilized CNPs on SPCE strips. This is because the Dengue antigen was unable to form an antigen-antibody non-conductive layer with the antibody of JEV.

In order to fabricate a more reliable and sensitive biosensor strips, further investigation on the fabrication of hybrid AuNPs and the chitosan CNPs will be discussed in the Chapter 5.

CHAPTER 5

FABRICATION OF HYBRID GOLD-CARBON NANOPARTICLES (Au-CNPs) BY SELF ASSEMBLY METHOD FOR *JAPANESE ENCEPHALITIS VIRUS* (JEV) ELECTROCHEMICAL BIOSENSOR

5.1 Introduction

Research on electrochemical biosensors with a typical platform for parts of biosensors including semi-conductor and screen-printed electrodes has been an important topic among researchers for a long time due to the wide range of applications in different areas (Saylan et al., 2019). Zhu et al. (2018) mentioned biosensors have been classified into four major groups which include potentiometric, amperometric, cyclic voltammetry, and impedimetric transducers by monitoring biosensor altering the dielectric properties, dimension, shape, and charge distribution where formation of antibody-antigen complex on the electrode surface.

Asadian et al. (2019) reported that carbon-based nanoparticles have shown a tremendous potential for wide range of electrochemical applications from energy storage to electrochemical biosensing due to intrinsic electrochemical activity, large specific surface area, electrocatalytic properties, facilitated electron transfer, and outstanding electrical conductivity. However, Sharma et al. (2010) have further proven gold nanoparticle to be an important material used in biomedical applications due to its various optical properties needed for biosensor applications with different sizes of nanoparticles especially in cancer nanotechnology. Furthermore, gold nanoparticles were mostly used in amplifying signals (Wang et al., 2015). According to the research conducted by Wang et al. (2015), amount of gold nanoparticles is measured through electrochemical method and hence determine the presence and amount of bacteria using biosensor coated with gold nanoparticles and it has

successfully reduced the detection time from 3 to 4 days to less than 1 h with a simple target extraction method.

Jazayeri et al. (2016) had further proven in their studies, the tremendous interest from researchers on gold nanoparticles is due to its unique surface, chemical inertness, high electron density, and strong optical absorption. These characteristics made to be applied in genomics, vaccine development, immunoassay, biosensors, diagnosis and microorganisms' control, cancer-cell imaging and drug delivery. Jazayeri et al. (2016) demonstrated on the physical interaction between antibodies and AuNPs which depends on three phenomena that includes ionic attraction between negatively charged gold and positively charged antibody, hydrophobic attraction between antibodies and gold surface and dative bond between gold conducting electrons and amino sulphur atoms of antibody.

Due to the special characteristics of carbon nanoparticles especially their optical and electrochemical properties which turn them into potential candidates for bioimaging, biosensing, drug delivery, and photodynamic applications (Asadian et al., 2019) and most importantly carbon nanoparticles are easier to synthesize, environmental friendly, energy saving, low cost (Sharma et al., 2010); the addition of gold nanoparticles (AuNPs) with CNPs produces the hybrid Gold-Carbon nanoparticles (Au-CNPs) which minimizes the limitations as well as providing additional synergistic properties, they are, higher effective surface area, catalytic activity, electrical conductivity, water solubility, and biocompatibility (Khalil et al., 2016). Therefore, it is highly expected that extraordinary outputs can be achieved when using hybrid gold-carbon nanoparticles (Au-CNPs) as the platform substrate for electrochemical biosensors and in others numerous applications as well (Daraee et al., 2016).

Despite all the advantages, the commercialization of carbon-based electrochemical biosensors remains as a challenge due to its stability, sensitivity and complex instrumentation design. Therefore, in this study, chitosan CNPs prepared in Chapter 3 and further immobilised with JEV antibody in Chapter 4 will be further modified with gold nanoparticles (AuNPs) to fabricate hybrid gold-carbon nanoparticles (Au-CNPs) modified SPCE for detection of Japanese Encephalitis Virus (JEV). Performances of immunosensor are investigated *via* different experimental conditions include various size of gold nanoparticles (10 nm, 20 nm and 40 nm) fabricated with Chitosan CNPs and electrical conductivity studies for different ratio of CNPs versus AuNPs. In addition, electrochemical characterization in term of their selectivity, capability and sensitivity performance biosensor strip based on hybrid gold-carbon nanoparticles (Au-CNPs) modified SPCE for detection of JEV is also performed in this study.

5.2 Materials and Methods

5.2.1 Chemicals

All chemicals were of reagent grade and used without further purification. Sodium tripolyphosphate (TPP) of technical grade (85%), Bovine Serum Albumin (BSA), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide 98% (NHS) were supplied by Sigma-Aldrich (St. Louis, USA). Acetic acid was from HmbG Chemicals (Hamburg, Germany), Chitosan powder with molecular weight of 100–300 kDa was purchased from Acros Organics (New Jersey, USA), Sulphuric acid of technical grade (95%) was purchased from J.T.Baker (Thailand), Phosphate buffer saline (PBS, pH 7) and Gold nanoparticles (Au-NPs) in 10 nm, 20 nm and 40 nm stabilized suspension in citrate buffer were obtained from Merck (Germany), Acetic acid glacial ($C_2H_4O_2$), Potassium ferricyanide ($K_3[Fe(CN)_6]$) and potassium ferrocyanide ($K_4[Fe(CN)_6]$)

were obtained from Bendosen (Bendosen Laboratory Chemicals, Bendosen, Norway). Screen printed carbon electrodes (SPCE) with a carbon-based working electrode, carbon-based counter electrode, and a silver/silver chloride (Ag/AgCl) reference electrode were purchased from a local vendor Rapid Genesis Sdn Bhd. Ultra-pure water (18.2 MΩ.cm) was obtained from a water purifying system (ELGA Model: Ultra Genetic). JEV specific monoclonal antibody (Clone ID: MV12/1/C2–2/1), JEV antigen, JEV positive and JEV negative human serums (Dengue antigen) clinical samples were provided by the Institute of Health and Community Medicine (IHCM), Universiti Malaysia Sarawak and these biological products were stored at - 20 °C.

5.2.2 Preparation of Hybrid Gold-Carbon Nanoparticles (Au-CNPs)

Hybrid gold-carbon nanoparticles were prepared by mixing 100 µL of Carbon nanoparticles (CNPs) formulated from preformed Chitosan nanoparticles as reported in Chapter 4, Section 4.2.2 with 100 µL each of 10 nm, 20 nm and 40 nm gold nanoparticles (Au-NPs) solution purchased from Merck (Ratio CNPs : AuNPs of 1:1) through self-assembly method (SAM). The mixtures were under gently shaking for 2 hr and then incubated in room temperature for 24 hr in dark condition. To remove unbound Au-NPs, the solution was centrifuged at 13,200 rpm for 20 min and the black colour of hybrid 10 nm gold-carbon nanoparticles (Au10-CNPs), hybrid 20 nm gold-carbon nanoparticles (Au20-CNPs) and hybrid 40 nm gold-carbon nanoparticles (Au40-CNPs) produced were then rinsed and dried in a vacuum desiccator for 1 day.

5.2.3 Characterization of Hybrid Gold-Carbon Nanoparticles (Au-CNPs)

Field Emission Scanning Electron Microscope (FESEM) (JSM – IT500 HR), Transmission Electron Microscope (TEM, JEOL Model JEM-1230), and energy dispersive

X-ray (EDX) spectroscopy were used to characterize the surface morphology and structure of Au10-CNPs, Au20-CNPs and Au40-CNPs. The absorption spectra of the samples (CNPs, AuNPs and Au-CNPs) were measured with a UV-Vis Spectrophotometer (Shimadzu UV-160A) to confirm the successful fabrication of hybrid gold-carbon nanoparticles (Au-CNPs).

However, CV and EIS were used for the electrochemical characterisation of various type of hybrid gold-carbon nanoparticles (Au10-CNPs, Au20-CNPs and Au40-CNPs). CV and EIS measurements were performed on a Potentiostat (Princeton Applied Research, PARSTAT 2263, using an electrolyte containing 15.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl. For CV characterization, the potential was cycled between -1.3 V and $+1.0$ V (vs Ag/AgCl) at a scan rate of 50.0 mVs^{-1} . The EIS measurements were recorded within the frequency range of 0.01 Hz to 100.0 kHz.

5.2.4 Optimization of Experimental Parameters

Optimum condition of experimental parameter is crucial to produce high performance of electrochemical biosensor. Further investigation of the optimized amount of AuNPs loading on CNPs based on their electrochemical performance by using electrochemical impedance spectroscopy (EIS) is carried out on the selected size of hybrid 10 nm gold-carbon nanoparticles (Au10-CNPs) with various manipulated ratio of AuNPs versus constant of CNPs (Ratio 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 and 10:1. In addition, effect of the temperature and response time of the immunosensor also being investigated in this studies.

5.2.5 Activation of the JEV antibody and Immobilizing on Surface of Au10-CNPs

A monoclonal antibody (Clone ID: MV12/1/C2-2/1), raised in a mouse specific to produce immune response to JEV, and originally provided by Venture Technology, Penang,

Malaysia (Cardosa et al., 2002; Sum et al., 2015), was pre-treated with ethyl(dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) for activation. 200 μ L each of ethyl(dimethylaminopropyl) carbodiimide (EDC, 0.2 M) and N-Hydroxysuccinimide (NHS, 0.05 M) were added to 200 μ L of JEV antibody (10.0 M) to activate its carboxyl groups by allowing them to react for 1 hour at 4 $^{\circ}$ C. The activated JEV antibody was then added onto the surfaces of Au10-CNPs at room temperature and then incubated for 24 hours to immobilize the JEV antibody on Au10-CNPs surfaces by allowing formation of amide bonds between amino groups on CNPs surface and activated carboxyl groups on the JEV antibody.

5.2.6 Fabrication of Hybrid Au10-CNPs Modified SPCE Electrochemical Biosensor

A total of 10 μ L of JEV antibody immobilised Au10-CNPs was drop-coated onto the working electrode of the SPCE strip and incubated for 48 hours at 37 $^{\circ}$ C *via* physically binding mechanism and then 2% (w/v) bovine serum albumin (BSA) in PBS was then added onto the surface of the working electrode to block unsaturated and non-specific binding sites. The electrode was rinsed with PBS to remove excess BSA.

5.2.7 Detection of JEV Antigens

For detection of JEV antigens, 10 μ L of JEV of various concentrations ranging from 1 to 20.0 ngmL $^{-1}$ was dropped onto the Hybrid Au10-CNPs modified SPCE electrochemical biosensor strip, incubated at 37 $^{\circ}$ C for 20 min, rinsed and air-dried. The interaction of JEV with antibody immobilized on the surface of CNPs modified SPCE electrochemical biosensor strip working electrode was studied using EIS. The relative resistance was calculated based on the equation discussed earlier at Chapter 4, Equation 4.1 (Rezaei et al., 2011). R_{et} (i) is the value of electron transfer resistance after the binding of JEV to JEV

antibody while R_{et} (o) is the value of electron transfer resistance after the immobilization of JEV antibody onto Au10-CNPs modified SPCE electrochemical biosensor strip working electrode surface and block with BSA. Figure 5.1 below illustrated the schematic diagram of preparation processes employed on the SPCE strip for fabricating Au-CNPs-SPCE electrochemical biosensor for the detection of JEV.

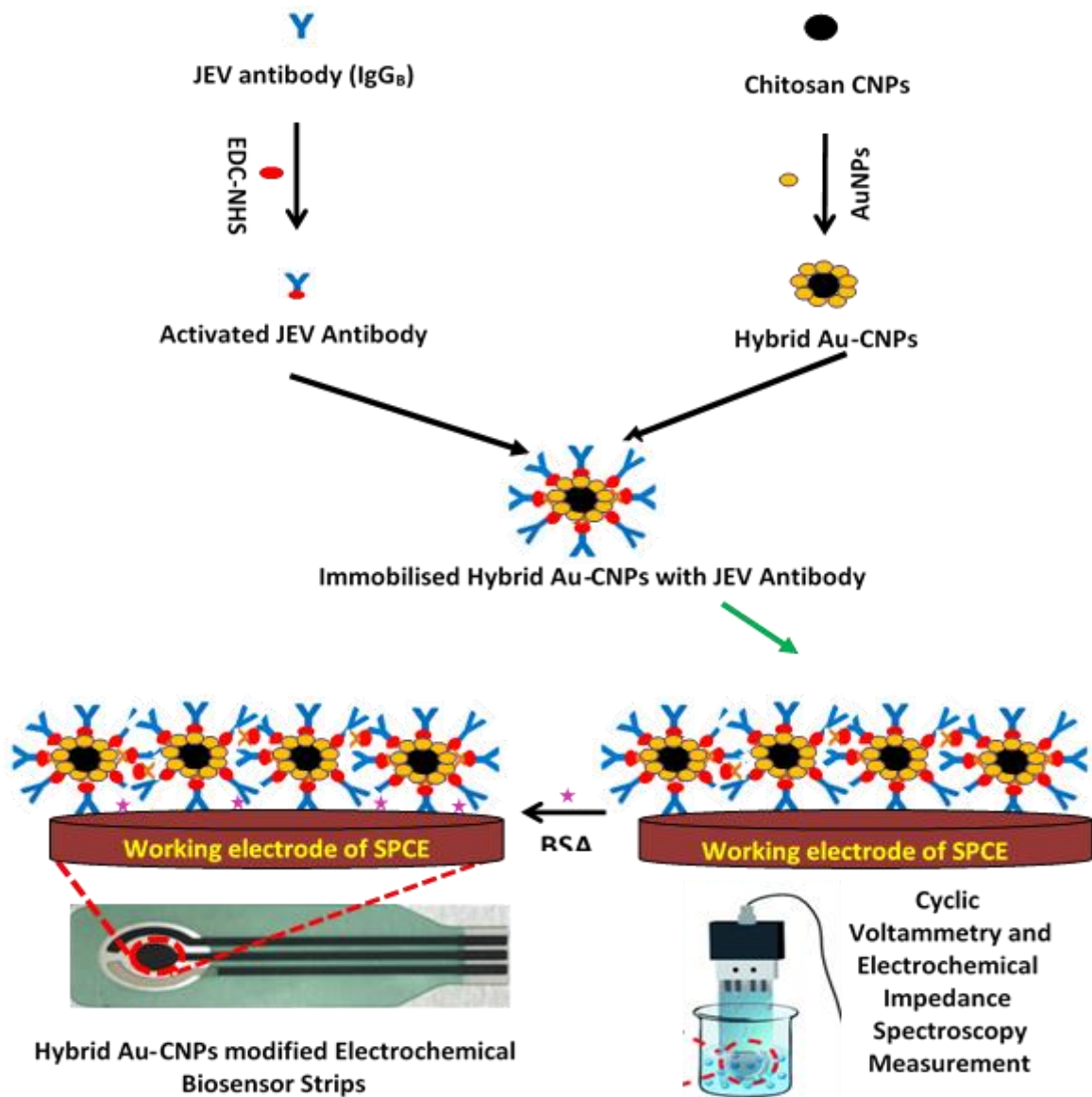


Figure 5.1: Schematic diagram of preparation processes employed on the SPCE strip for fabricating electrochemical biosensor for the detection of JEV (Lai et al., 2017).

5.3 Results and Discussion

5.3.1 Characterization of Hybrid Gold-Carbon Nanoparticles (Au-CNPs)

5.3.1.1 Ultraviolet-visible Spectrophotometer (UV-Vis)

UV-Vis absorption spectra for chitosan carbon nanoparticles (CNPs), colloidal gold nanoparticles solution (AuNPs), hybrid 10 nm gold-carbon nanoparticles (Au10-CNPs), hybrid 20 nm gold-carbon nanoparticles (Au20-CNPs) and hybrid 40 nm gold-carbon nanoparticles (Au40-CNPs) are shown in Figure 5.2. Before AuNPs immobilized with CNPs, peak appeared at 520 nm which was caused by the plasmon resonance on the surface and comparing the UV-Vis spectrum between the AuNPs with hybrid gold-carbon nanoparticles (Au-CNPs), it is clearly showed the decreasing of the intensity of surface plasmon resonance at 520 nm and it can also determine that the smaller the size of the AuNPs, the greater the intensity loss at 520 nm. Since the decreasing of intensity is only found after the AuNPs were deposited, which indicates that the Au10-CNPs could be successfully adsorbed and firmly attached onto the surface of the carbon nanoparticles compare to bigger size of Au-CNPs. It can be confirmed when compared the various sizes of hybrid gold-carbon nanoparticles (Au-CNPs) with the spectrum of carbon nanoparticles (CNPs), a new absorption band centred at 352 nm is observed in the spectrum of the hybrid 10 nm gold-carbon nanoparticles (Au10-CNPs), hybrid 20 nm gold-carbon nanoparticles (Au20-CNPs) and hybrid 40 nm gold-carbon nanoparticles (Au40-CNPs) which indicates the capture of AuNPs by CNPs. The shift in the resonance wavelength was attributed to interparticle plasmon coupling (Cui et al., 2008a; Cui et al., 2008b; Cui et al., 2013 and Cui et al., 2018).

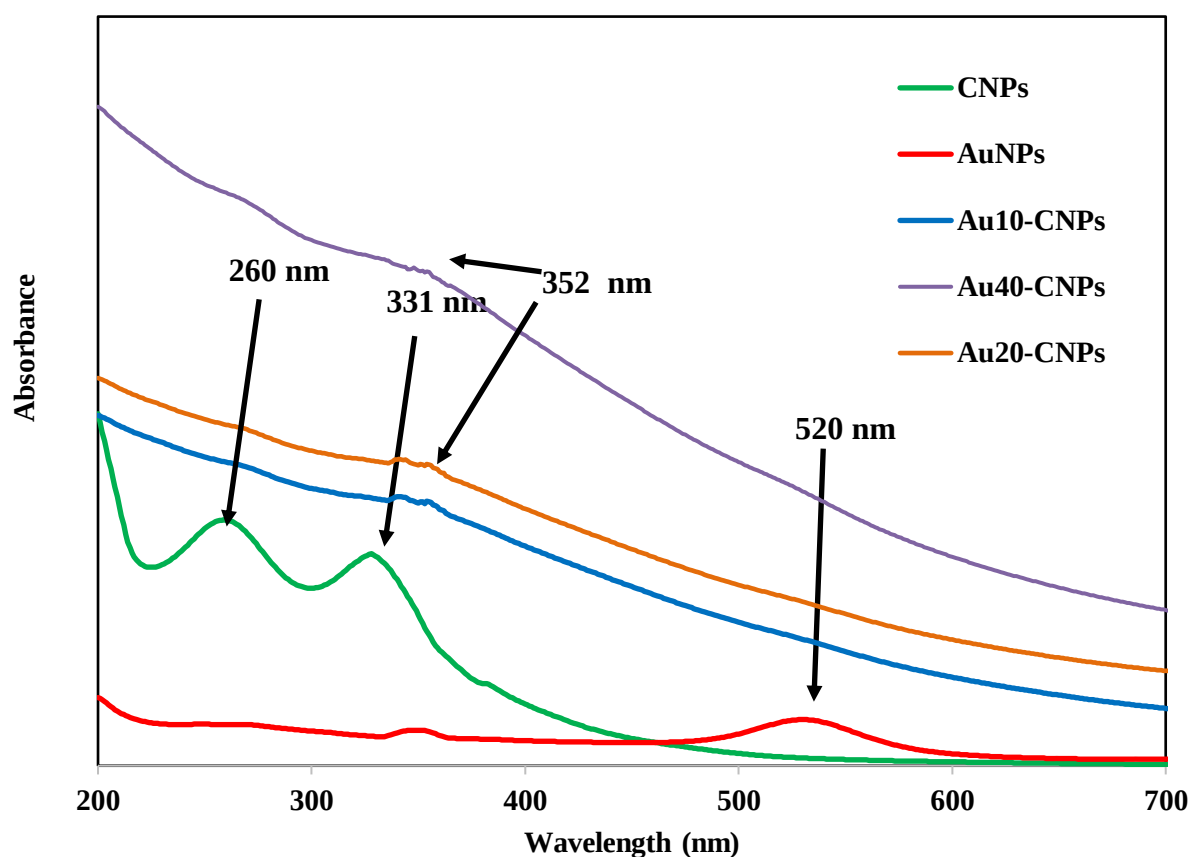


Figure 5.2: UV-Vis absorption spectra of the chitosan carbon nanoparticles (CNPs), colloidal gold nanoparticles solution (AuNPs), hybrid 10 nm gold-carbon nanoparticles (Au10-CNPs), hybrid 20 nm gold-carbon nanoparticles (Au20-CNPs) and hybrid 40 nm gold-carbon nanoparticles (Au40-CNPs).

5.3.1.2 Transmission Electron Microscope (TEM)

Figure 5.3 (A) to (C) showed the typical transmission electron micrograph (TEM) for the materials of pure colloidal gold nanoparticles (AuNPs) with various size of 10 nm, 20 nm and 40nm were used for the preparation of hybrid gold-carbon nanoparticles (Au-CNPs). The TEM images give a direct vision of the gold nanoparticles immobilized onto carbon nanoparticles (CNPs) as shown in Figure 5.3 (D) –(F).

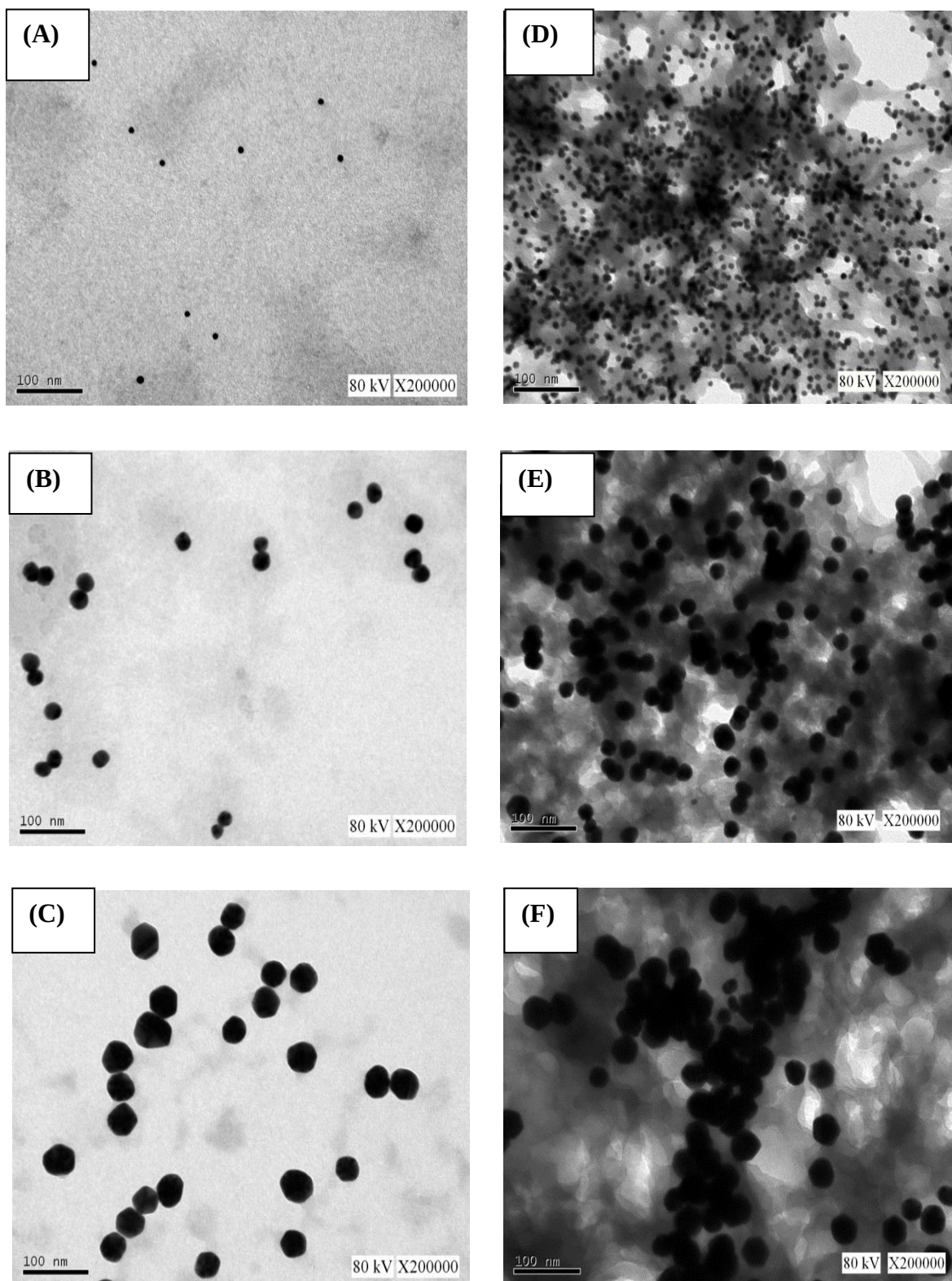


Figure 5.3: Transmission electron micrographs of pure colloidal gold nanoparticles (AuNPs) of (A) 10 nm AuNPs, (B) 20 nm AuNPs, (C) 40 nm AuNPs; and hybrid gold-carbon nanoparticles (Au-CNPs) of (D) Au10-CNPs, (E) Au20-CNPs and (F) Au40-CNPs.

Deposited gold nanoparticles (AuNPs) were in spherical shape; and were observed to be distributed evenly, scattered well and firmly attached to the CNPs surface which can be concluded that the hybrid gold-carbon nanoparticles for Au10-CNPs, Au20-CNPs and Au40-CNPs *via* self-assembly method (SAM) were successfully synthesized. However, further investigation on the TEM images for the morphology structure of the hybrid gold-carbon nanoparticles (Au-CNPs) found that the smallest size (10 nm AuNPs) gold nanoparticles (Figure 5.4) with its larger total surface area properties distributed on the CNPs were much better than Au20-CNPs and Au40-CNPs and it had better potential to contribute a larger surface area for further modification or functionalization for the fabrication of greater performance of electrochemical biosensor.

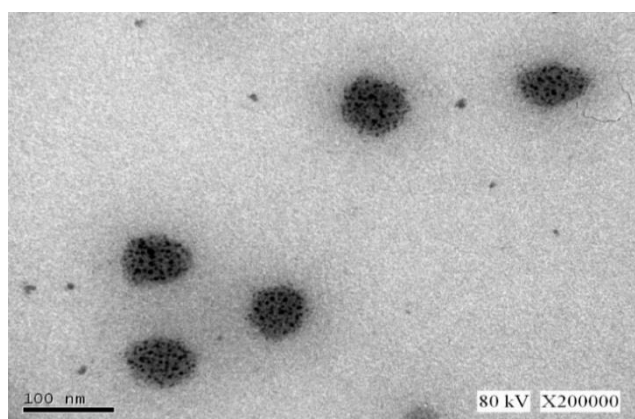


Figure 5.4: Transmission electron micrographs (TEM) of hybrid 10 nm gold-carbon nanoparticles (Au10-CNPs).

5.3.1.3 Field Emission Scanning Electron Microscope (FESEM) and Energy Dispersive X-ray (EDX) spectroscopy

The presence of AuNPs on the CNPs surface were also supported by the FE-SEM micrograph and EDX spectrum as reported in Figure 5.5 and the percentage (% wt.) of gold elements detected ranging from 0.52% wt. to 2.63% wt. which is inversely proportional to the size of AuNPs. The high percentage of weight for carbon and oxygen element were

observed in EDX spectrum and this might be due to the high percentage of carbon and oxygen atoms per molecule in chitosan.

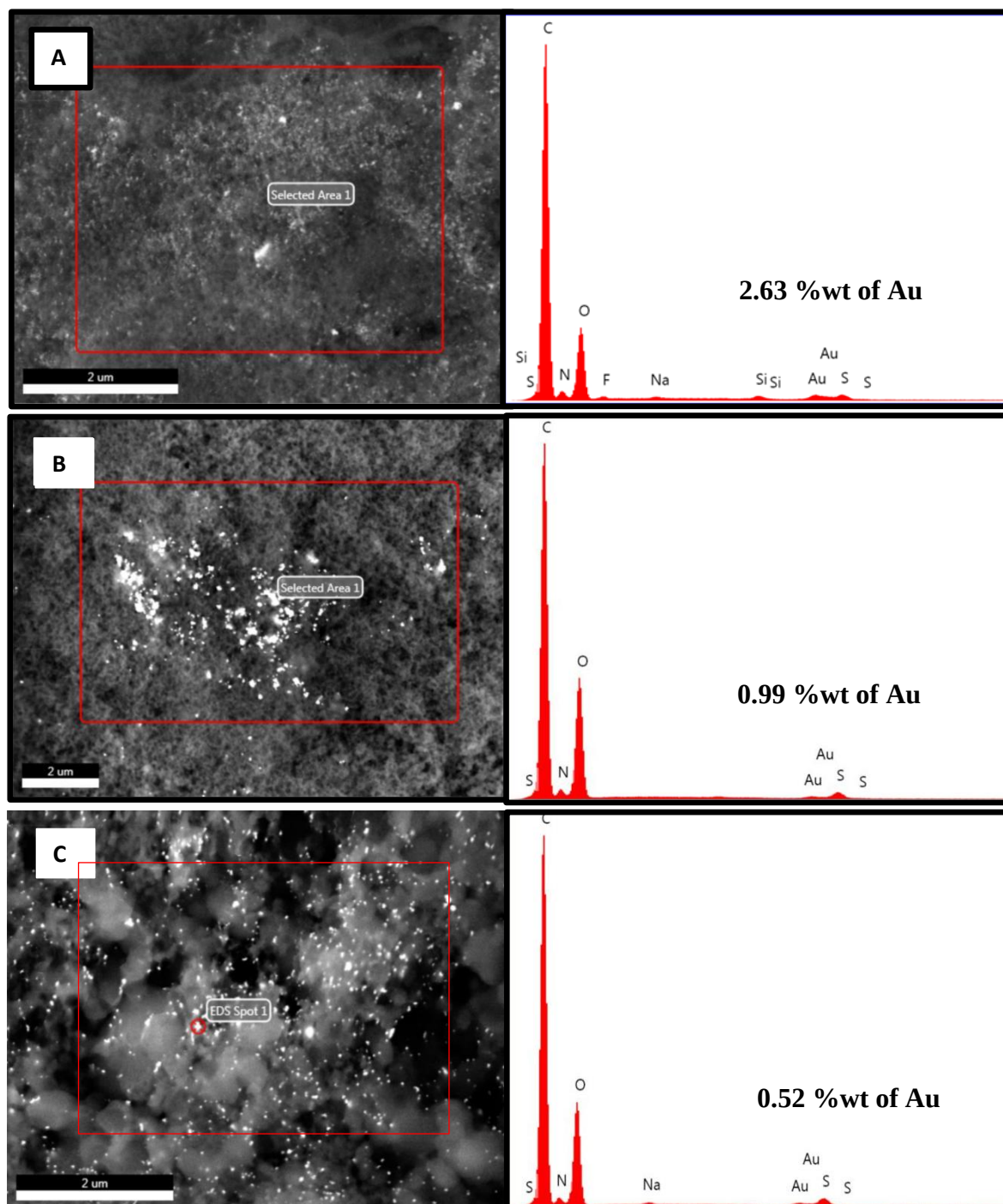


Figure 5.5: FESEM (left) images and energy dispersive X-ray (EDX) spectrum (right) of hybrid gold-carbon nanoparticles for (A) Au10-CNPs, (B) Au20-CNPs and (C) Au40-CNPs.

5.3.1.4 Cyclic Voltammetry (CV)

Cyclic voltammetry (CV) with $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox pair is informative in determining the electrochemical properties of the electrode surface; it is also suitable to characterize the electrochemical responses or electrical conductivity properties of hybrid gold-carbon nanoparticles (Au-CNPs) fabricated using different sizes (10 nm, 20 nm and 40 nm) of gold nanoparticles (AuNPs) in the presence of 15 mM $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (1:1) as redox probes and 0.1 M KCl at pH ~ 7.4 . Cyclic voltammogram in Figure 5.6 showed that hybrid gold-carbon nanoparticles (Au-CNPs) modified SPCE electrochemical biosensor strip (light blue, purple and dark blue curve) exhibited higher both anodic and cathodic current density than bare SPCE strip (red curve). The presence of hybrid gold-carbon nanoparticles (Au-CNPs) led to an increase in the conductivity and effective electroactive surface area of SPCE.

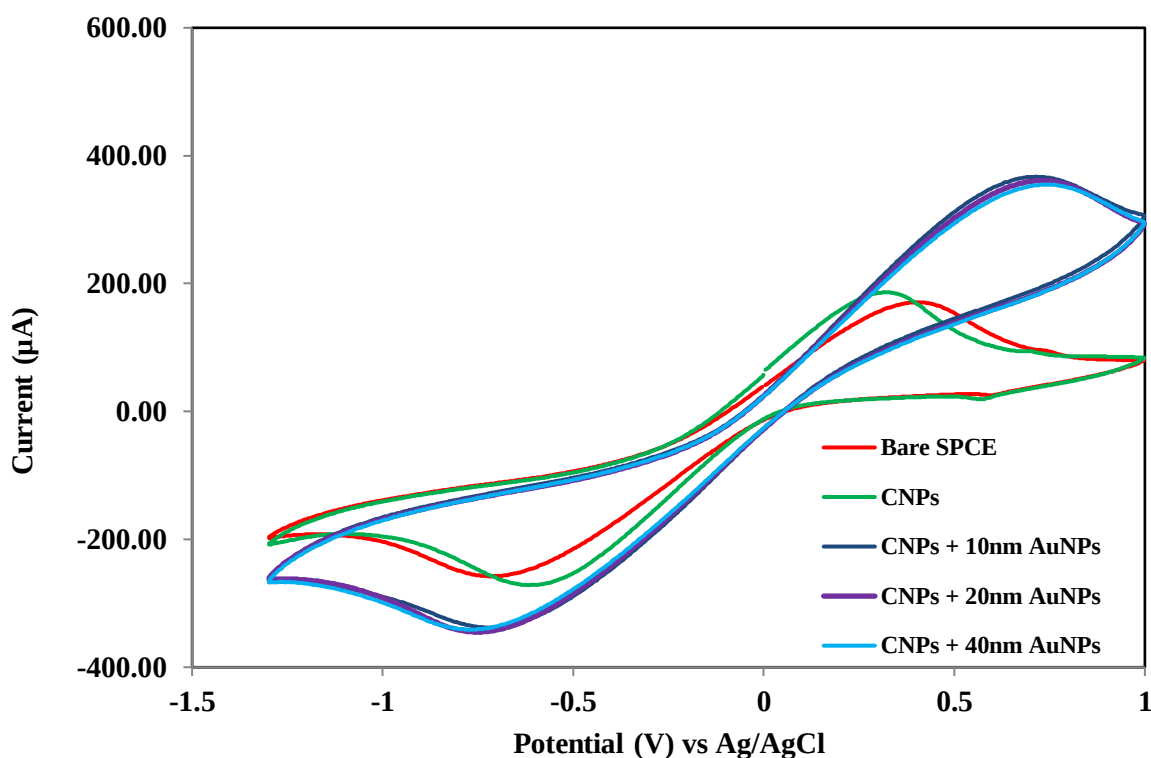


Figure 5.6: CV of bare SPCE strip, CNPs SPCE strip, Au10-CNPs on SPCE strip, Au20-CNPs on SPCE strip, Au40-CNPs on SPCE strip.

5.3.1.5 Electron Impedance Spectroscopy (EIS)

Electron impedance spectroscopy (EIS) has been reported as an effective method to monitor the features of a surface to allow the understanding of chemical transformations and transfer of electron with the conductive electrode surface (Cui et al., 2008a; Cui et al., 2018). The impedance spectra include a semicircle portion and a linear portion, the semicircle portion at higher frequencies corresponds to the electron transfer limited process, and the linear part at lower frequencies corresponds to the diffusion process. The semi-circle diameters correspond to the electron-transfer resistance (R_{et}) and all EIS data were simulated by the Randless equivalent circuit as shown in the inset of Figure 5.7. As observed in the Nyquist plot of bare SPCE electrochemical biosensor strip displayed a larger semicircle of larger diameter (red curve) than that of hybrid gold-carbon nanoparticles (Au-CNPs) modified SPCE electrochemical biosensor strip (light blue, purple and dark blue curve). Bare SPCE had exhibited a higher electron transfer resistance ($R_{et} = 764 \text{ ohm}$) as compared with Au10-CNPs modified SPCE ($R_{et} = 235.9 \text{ ohm}$) due to faster electron transfer rates at the electrode surface.

Therefore, based on the of characterization that had been done in Section 5.3.1.1 to Section 5.3.1.5, hybrid 10 nm gold-carbon nanoparticle (Au10-CNPs) will be the most promising to produce greater electrochemical biosensor. Therefore, Au10-CNPs will be chosen for further optimization and functionalisation process. Nevertheless, the sensitivity, selectivity and capability performance will be also demonstrated by using Au10-CNPs.

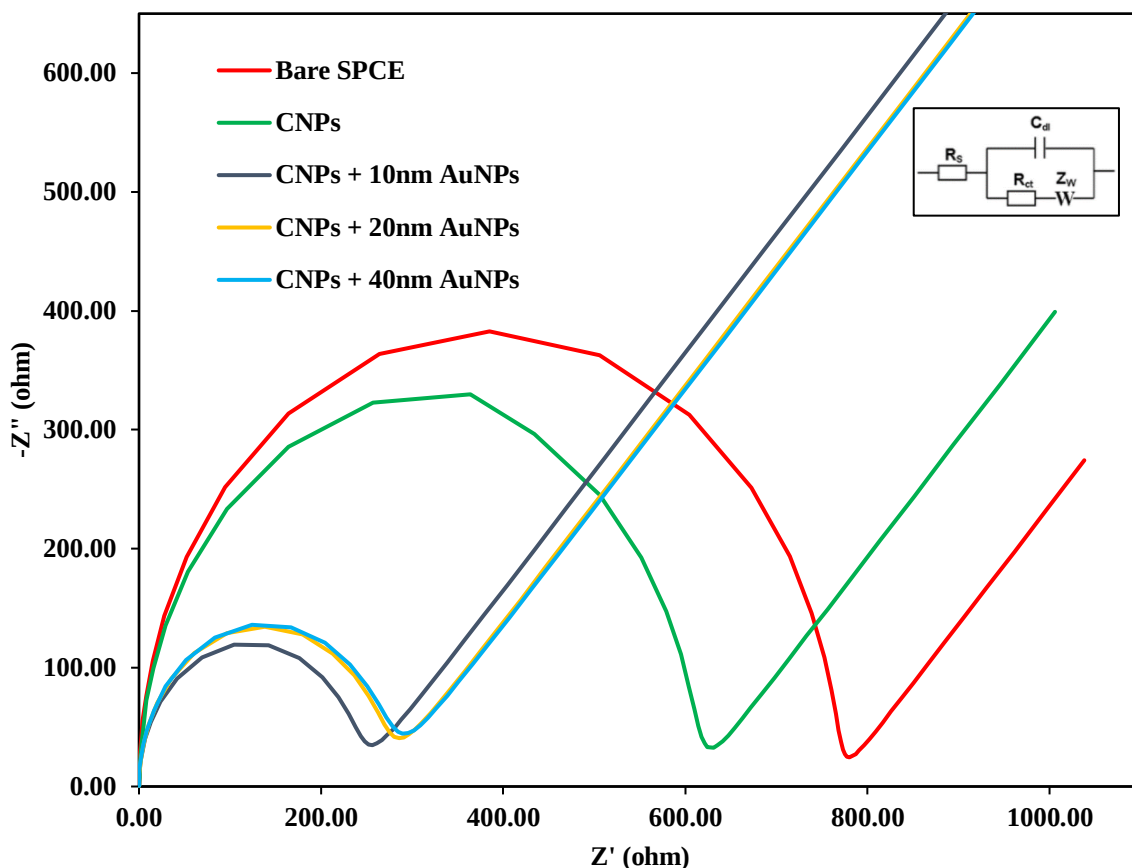


Figure 5.7: Nyquist plots of bare SPCE strip, CNPs SPCE strip, Au10-CNPs on SPCE strip, Au20-CNPs on SPCE strip, Au40-CNPs on SPCE strip. [Inset shows the Randle equivalent circuit]

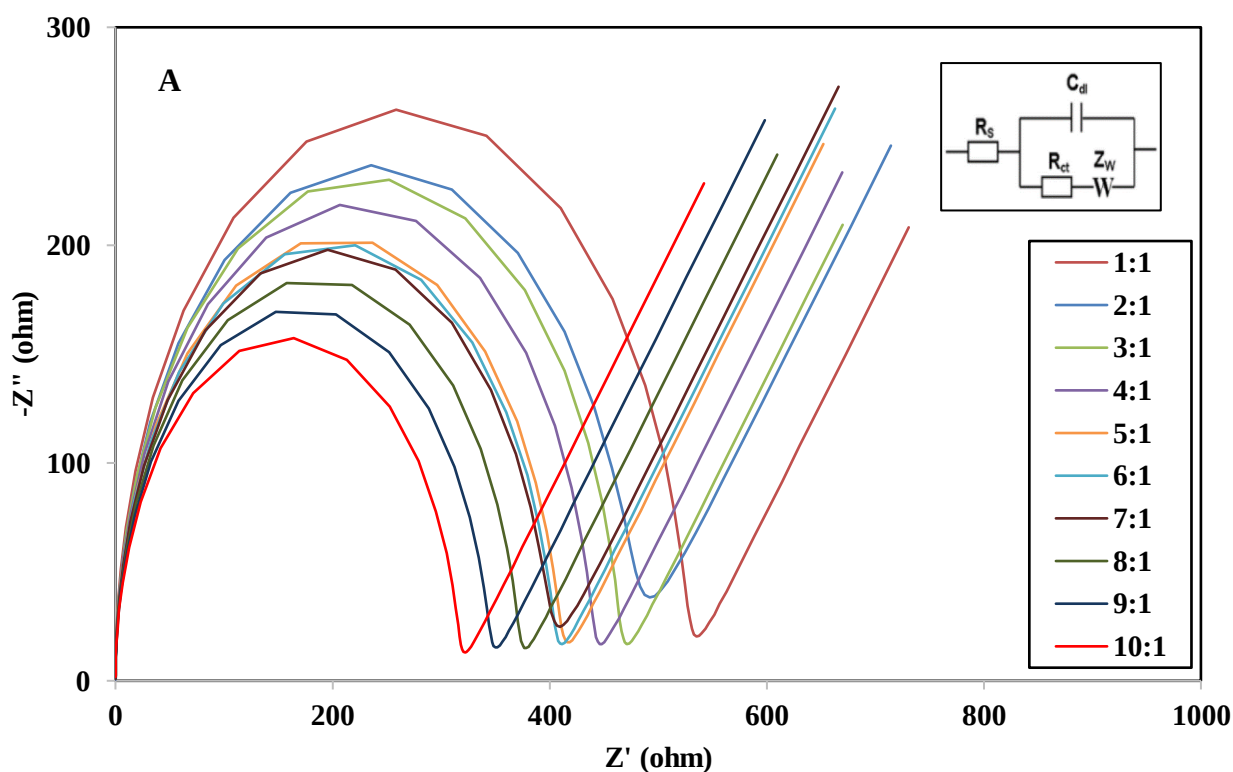
5.3.2 Optimization of Experimental Parameters

To fabricate greater performance and high sensitivity of the hybrid gold-carbon nanoparticles (Au-CNPs) modified SPCE biosensor for the detection of JEV antigen, various analytical parameters affecting the analytical signals and electrochemical performance were optimized. The experimental parameters in terms of amount of AuNPs loading on CNPs, temperature and response time of the Immunosensor were carried out in this study.

5.3.2.1 Effect for Amount of AuNPs Loading on CNPs

Selected size of hybrid 10 nm gold-carbon nanoparticles (Au10-CNPs) with various manipulated ratio of AuNPs versus constant of CNPs (Ratio 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1,

8:1, 9:1 and 10:1) were optimized based on their electrochemical performance by using electrochemical impedance spectroscopy (EIS). All EIS data were simulated by Randle's Equivalent Circuit model as shown in the inset of Figure 5.8 (A). This circuit includes the uncompensated resistance of the solution phase (R_s), Warburg impedance (Z_w) resulting from the diffusion of ions from bulk electrolyte to electrode surface, double-layer capacitance (C_{dl}), and electron-transfer resistance (R_{et}) which is inversely proportional to the rate of electron transfer (Piermarini et al., 2013). Based on the result as shown in Figure 5.8 (B), it is interesting to highlight that the higher the amount of AuNPs loaded to CNPs (Ratio AuNPs : CNPs = 10:1), the lower the value of electron transfer resistance ($R_{et} = 313.7 \text{ ohm}$), and yet, the higher the electrical conductivity on the electrode surface which showed linear correlation with $R_2 = 0.9737$. Thus, the higher the ratio of AuNPs : CNPs, the higher the number of the gold nanoparticles per unit volume and resulting in higher rate of electron transfer on electrode surface (Bhatnagar et al., 2017).



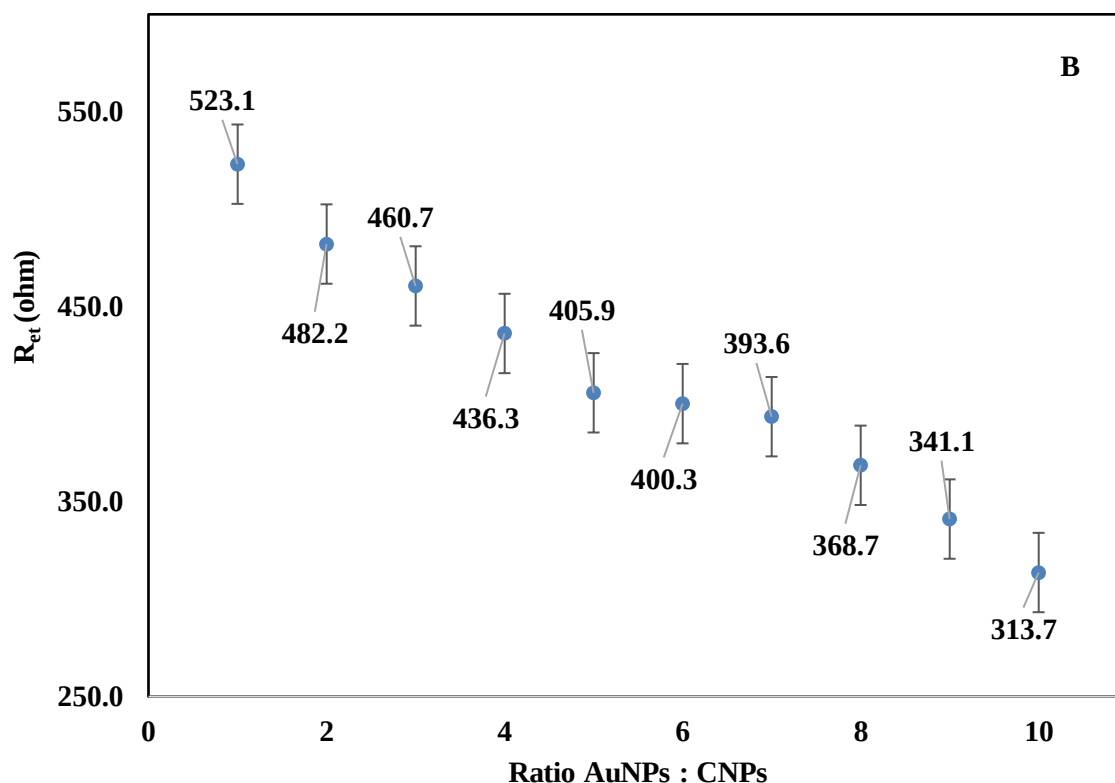


Figure 5.8: (A) Nyquist plots of ratio AuNPs:CNPs for 1:1; 2:1; 3:1; 4:1; 5:1; 6:1; 7:1; 8:1; 9:1 and 10:1 (B) Error bar shows electron transfer resistance, R_{et} for various Nyquist plot of Ratio AuNPs:CNPs (1:1; 2:1; 3:1; 4:1; 5:1; 6:1; 7:1; 8:1; 9:1 and 10:1). [Inset of 5.8 (A) shows the Randle Equivalent circuit.

5.3.2.2 Effect of Electrolyte Temperature

The effect of electrolyte temperature on the receptor target interaction was tested based on several manipulated temperature (10 °C, 20 °C, 35 °C, 45 °C, 60 °C and 80 °C). This study was tested based on their electrochemical performance by using electrochemical impedance spectroscopy (EIS). Based on the Nyquist plot shown at Figure 5.9 below, at the temperature of 10 °C, 60 °C and 80 °C, it showed a weak signal for the rate of electron transfer. However, at 35 °C, it showed a lowest electron transfer resistance ($R_{et} = 212.8$ ohm) significant to higher electrical conductivity on the electrode surface. Thus, for the practical purpose, we performed successive experiments at 35 °C.

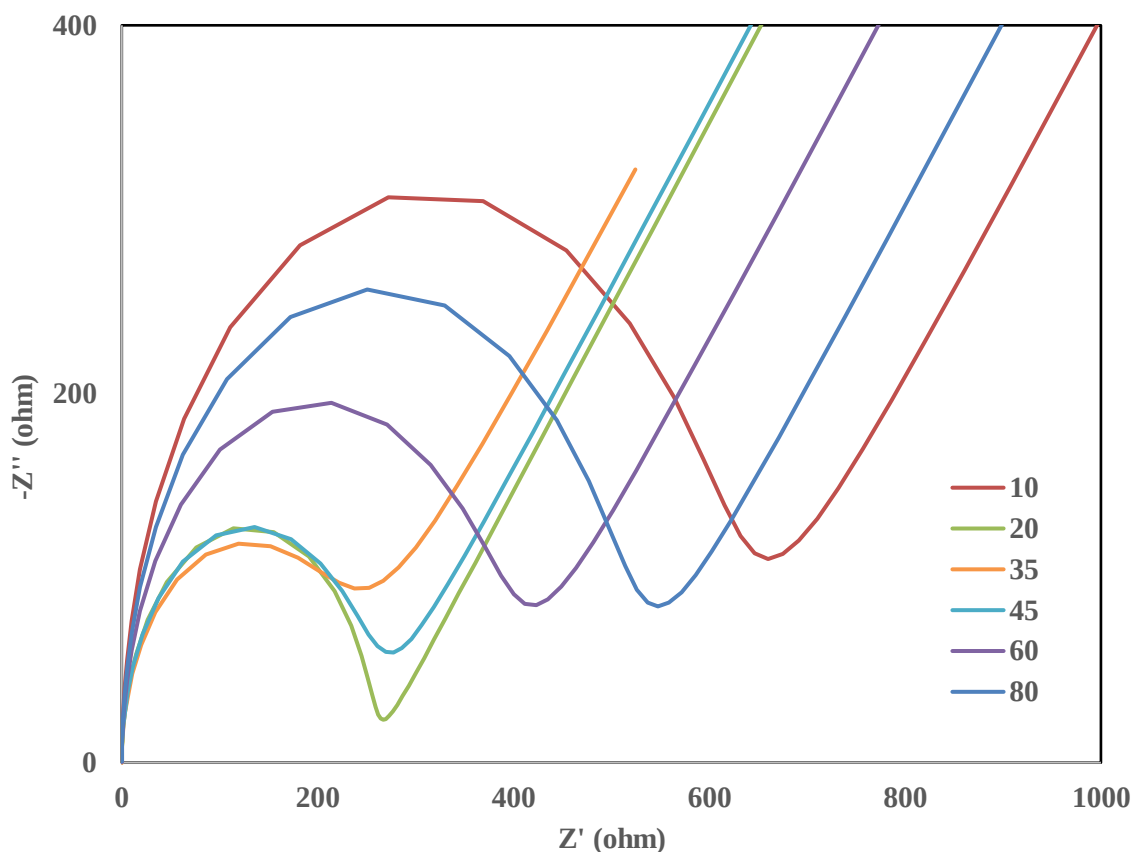


Figure 5.9: Nyquist plots of various electrolyte temperature of 10 °C, 20 °C, 35 °C, 45 °C, 60 °C and 80 °C.

5.3.2.3 Response Time of the Immunosensor

Cyclic voltammograms of Au10-CNPs modified SPCE electrochemical biosensor strip as a function of response time (1 min, 3 min, 5 min, 7 min, 10 min, 13 min, 15 min and 20 min) was shown in Figure 5.10. The Au10-CNPs modified SPCE electrochemical biosensor strip was observed to achieve stable cathodic current in about 10 minutes. This result demonstrated that the proposed biosensor strip exhibited rapid responses and results of the analysis were obtainable within 10 minutes. This observed response time is considerably faster than that reported for interdigitated biosensor at 20 minutes (Huy et al., 2011), and other JEV diagnosis methods which require diagnosis times ranging from hours to days (Taleat et al., 2014). The substantially enhanced sensitivity and faster response time could be attributed to the use of

Au10-CNPs which enabled a higher loading of JEV antibody as well as the increased overall electrical conductivity of the SPCE biosensor strip.

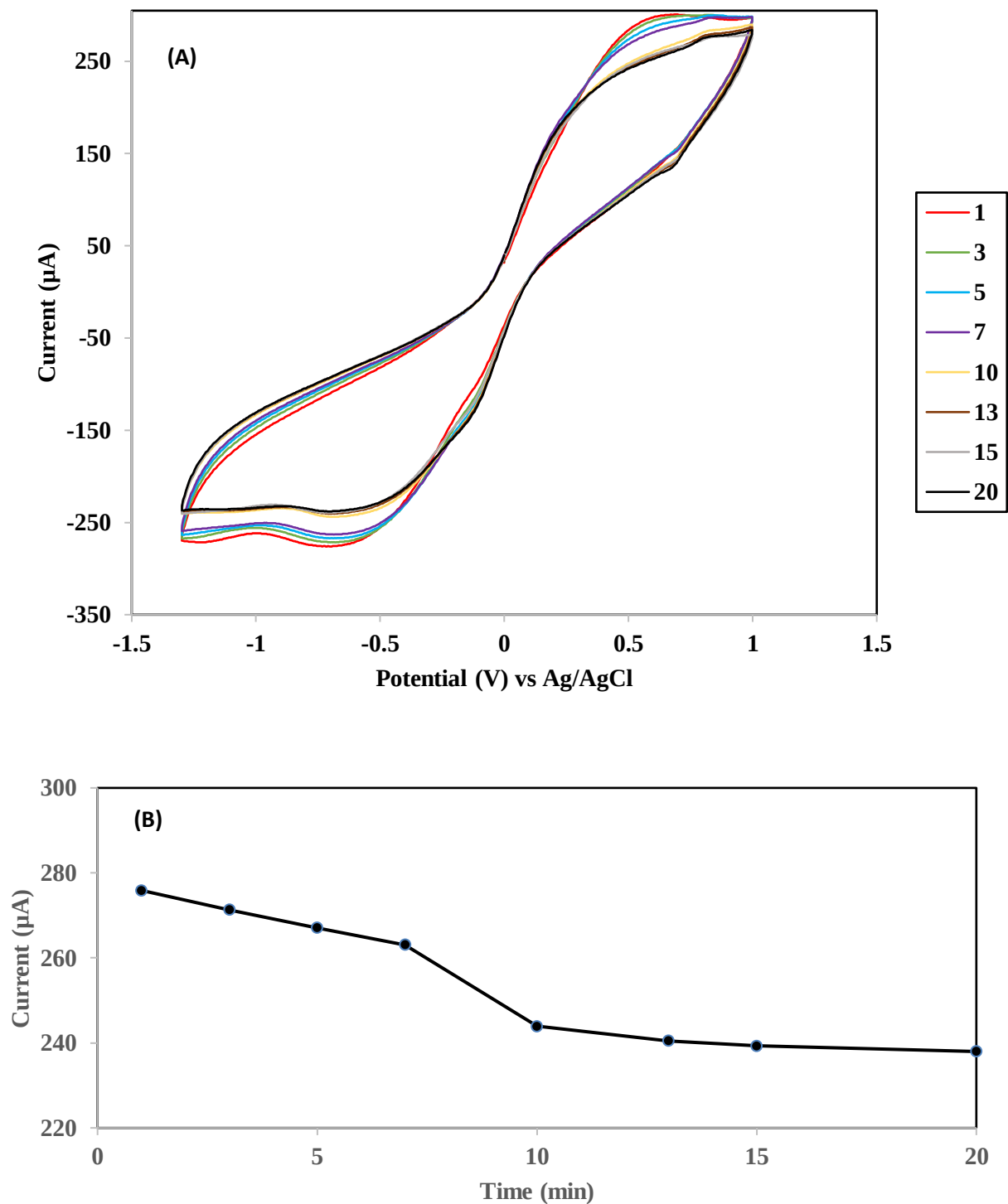


Figure 5.10: (A) Cyclic voltammograms of JEV antibody immobilized Au10-CNPs on SPCE biosensor strip as a function of response time: 1 min, 3 min, 5 min, 7 min, 10 min, 13 min, 15 min and 20 min and (B) Plot of current (μA) versus response time (min).

5.3.3 Specificity, Selectivity and Capability

In order to demonstrate clinical capability and selectivity of the Au10-CNPs modified SPCE electrochemical biosensor strip, both JEV positive human serum clinical samples and dengue antigen were used as the analytes and evaluated by using EIS under optimum condition of 35 °C and ratio AuNPs : CNPs with 10:1. As observed in Figure 5.11 in the presence of sample containing JEV positive human serum (curve v), the R_{et} (411.9 ohm) was observed to be substantially higher as compared to the R_{et} (309.6 ohm) of BSA blocked/JEV antibody immobilized Au10-CNPs on SPCE strips (curve iii). These results indicated the Au10-CNPs modified SPCE electrochemical biosensor strip was capable of JEV detection in human serums.

The selectivity of this biosensor strip was proven as in the presence of Dengue antigen, the R_{et} (288.9 ohm) of (curve iv) was observed to have no significant changes as compared with R_{et} (309.6 ohm) of BSA blocked/JEV antibody immobilized Au10-CNPs on SPCE strips (curve iii). This is because the Dengue antigen was unable to form an antigen-antibody non-conductive layer with the antibody of JEV.

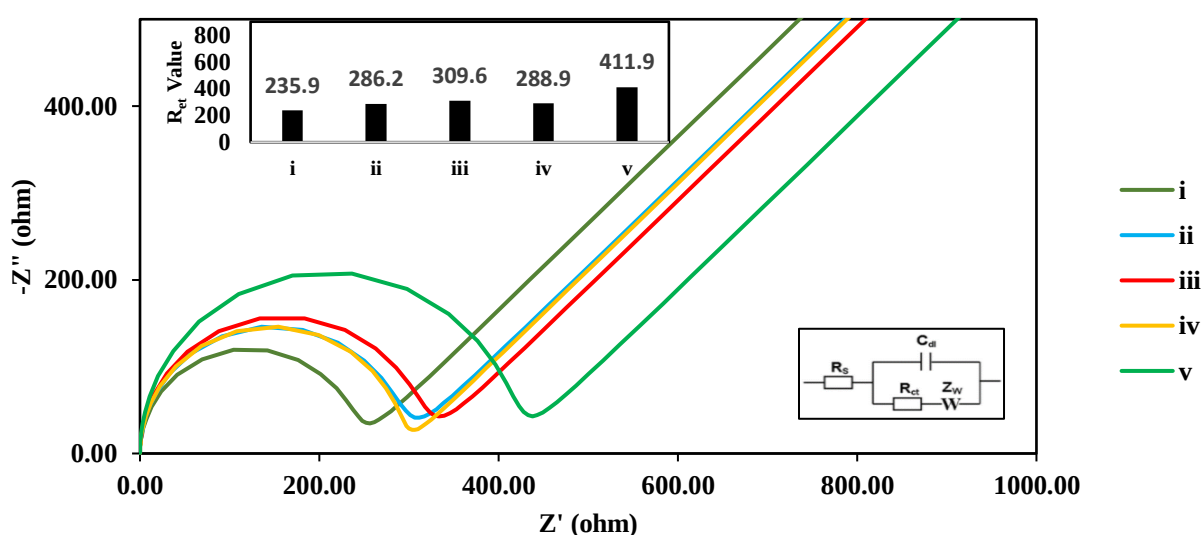


Figure 5.11: Nyquist plots of (i) Au10-CNPs SPCE strip; (ii) Au10-CNPs + JEV Ab on SPCE strip; (iii) Au10-CNPs + JEV Ab + BSA on SPCE strip; (iv) Au10-CNPs + JEV Ab + BSA + Dengue on SPCE strip; (v) Au10-CNPs + JEV Ab + BSA + Positive Serum [Inset (left) Chart shows electron transfer resistance, R_{et} for various Nyquist plot (i-v)]; [Inset (right) shows the Randle Equivalent circuit]

5.3.4 Sensitivity of Immobilised Hybrid Gold-Carbon Nanoparticles Modified on SPCE Electrochemical Biosensor Strip

The EIS spectra of Au10-CNPs modified SPCE electrochemical biosensor strip incubated with various concentrations of JEV was displayed in Figure 5.12. The diameters of semicircles were observed to increase as the concentration of JEV was increased. Figure 5.13 shows the calibration plots of relative resistance calculated by using equation 4.1 discussed earlier in Chapter 4 versus concentrations of JEV ranging from 1 to 20 ngmL⁻¹, the relative resistance increased linearly ($R^2 = 0.999$) with increased JEV concentration. The observed increase in R_{et} could be attributed to the formation of more antigen-antibody complexes at higher JEV concentration which inhibited the interfacial electron transfer process. A linear correlation equation between relative resistance and JEV concentration (ngmL⁻¹) obtained from Figure 5.13 as shown in Equation 5.1 below.

$$\text{Relative resistance} = 0.0404 \text{ Conc JEV} + 0.272 \quad \text{Equation 5.1}$$

Conc. JEV is the concentration of JEV in ngmL⁻¹. The value of the regression coefficient (R^2) and standard deviation (σ) were 0.99 and 0.27, respectively. The calibration plot of Relative Resistance versus concentrations of JEV ranging from 1 to 20 ngmL⁻¹ was used to determine the limit of detection (LOD) for the biosensor strip. The Au10-CNPs SPCE electrochemical biosensor strip showed a very low detection limit of 0.29 ngmL⁻¹ for JEV. From Equation 5.1, the detection sensitivity was 0.04 ngmL⁻¹. This detection limit is substantially lower than the previously reported detection limit of 10 ngmL⁻¹ for an interdigitated sensor (Tran et al., 2012b), a polyaniline nanowire-based electrochemical immunosensor at below 10 ngmL⁻¹ (Tuan et al., 2013) or my earlier studies reported 0.36 ngmL⁻¹ by using modified CNPs SPCE Strips (Lai et al., 2017). The Au10-CNPs modified

SPCE biosensor reported herein exhibited equivalent to more than an order of magnitude higher in sensitivity for JEV detection.

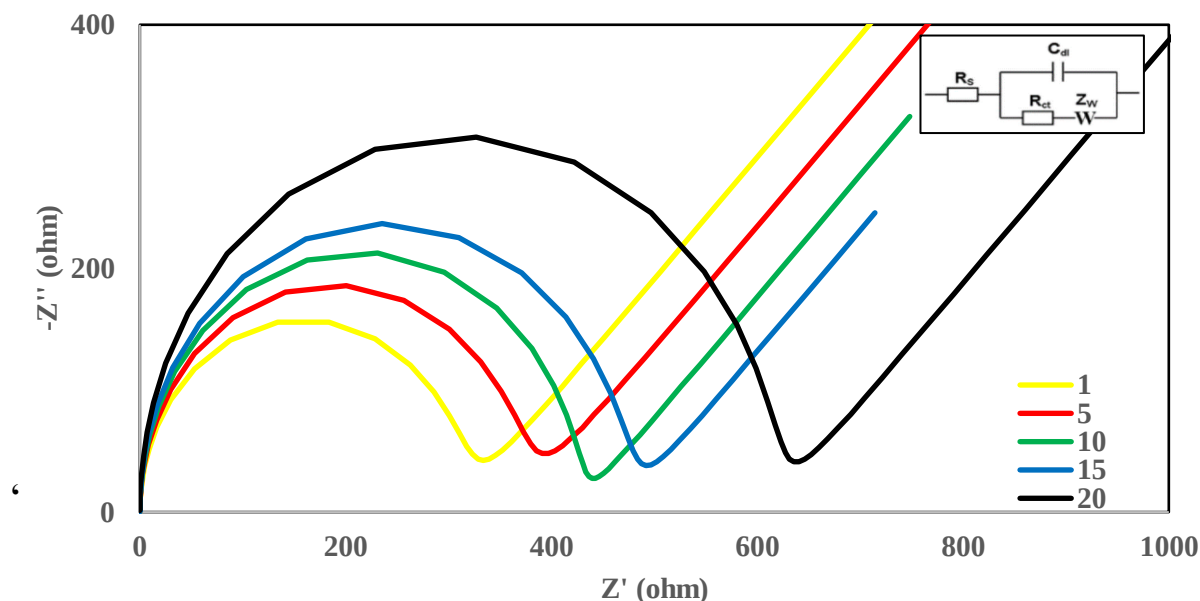


Figure 5.12: Nyquist plots of JEV antibody immobilized Au10-CNPs on SPCE strip with different JEV concentrations (ng/mL), Inset shows the Randle Equivalent Circuit.

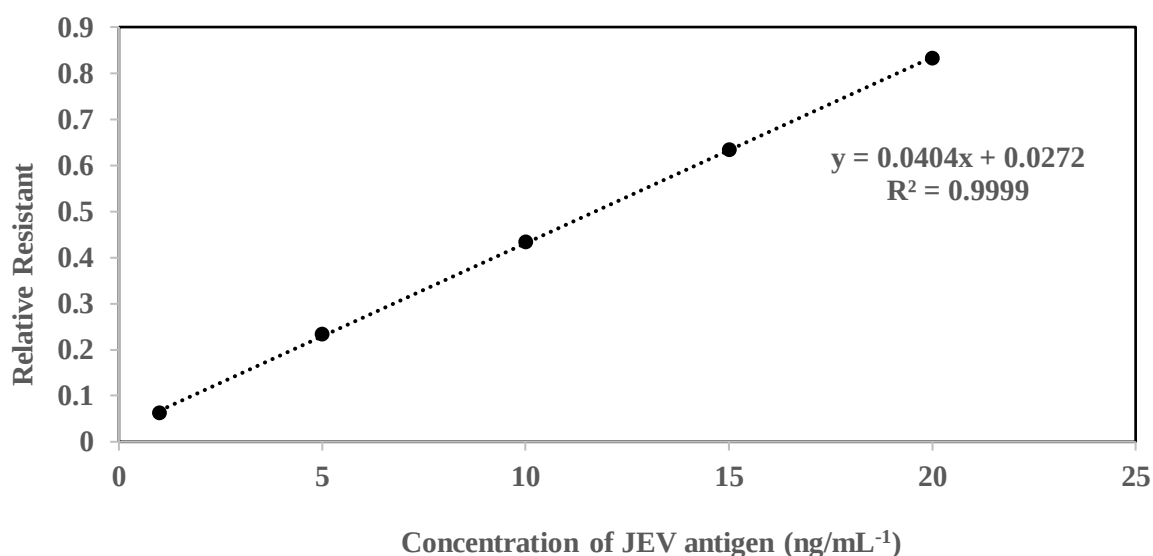


Figure 5.13: Line graph show calibration plots of relative resistance versus concentrations of JEV ranging from 1 to 20 ngmL⁻¹ with $R^2=0.999$ which were calculated from the mean value, $s/n = 3$

5.4 Conclusion

The novel hybrid gold-carbon nanoparticles (Au-CNPs) is successfully fabricated by the addition of successive chitosan CNPs synthesized in Chapter 3 with the immobilisation

reaction with JEV antibody that had been discussed in Chapter 4 and gold nanoparticles (AuNPs) by self-assembly methods.

The great performance of fabricated hybrid Au-CNPs can minimize the limitations by showing an extremely low LOD for the detection of JEV and as well as providing additional synergistic properties, higher effective surface area, catalytic activity, electrical conductivity, water solubility, and biocompatibility.

As expected, this novel biosensor strips exhibited rapid response and results of analysis were obtainable under 10 minutes and have shown a very low limit of detection of 0.29 ngmL^{-1} and detection sensitivity were 0.04 ngmL^{-1} for detection of JEV.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Concluding Remarks

From the experiments and findings reported from Chapter 3 to Chapter 5 in this thesis, several conclusions were drawn as follow.

CNPs of chitosan, starch, fibrous cellulose, carboxymethylcellulose, ascorbic acid and citric acid had been synthesized by refluxing with acid. The surface and morphology CNPs had been characterised by using FESEM and their performance of electrical conductivity of the CNPs had been analysed by using cyclic voltammetry (CV) and the findings CV is confirmed with electron-transfer resistance (R_{et}) value calculated from nyquist plot of EIS. Chitosan produced the smallest size of the CNPs ($\pm 89\text{ nm}$), the highest magnitude of current for both anodic and cathodic from cyclic voltammogram and the smallest electron-transfer resistance (R_{et}) calculated from nyquist plot of EIS. Results showed the faster electron transfer rates at the electrode surface and resulting in higher electrical conductivity.

As chitosan's functional properties of nontoxic, natural carbon sources, biocompatible, biodegradable, simple and rapid preparation process, optimization studies on reflux temperature were also being carried out and uncovered that reflux temperature of 180 °C can enhance the sensing performance.

From all the carbon-based nanoparticles synthesized using different types of precursor (carboxymethylcellulose (CMC), starch, citric acid, fibrous cellulose, chitosan, and ascorbic acid), chitosan CNPs with the optimum reflux temperature of 180 °C that

reported a greatest electrical conductivity performance is chosen to undergo immobilisation reaction with JEV antibody for JEV detection that had been discuss in Chapter 4.

In this work, modification of a disposable CNPs modified SPCE electrochemical biosensor strip was successfully fabricated for rapid and sensitive detection of JEV. This biosensor strip can achieve stable response signal within 10 minutes and has shown a low LOD of 0.36 ngmL^{-1} and detection sensitivity were 0.024 ngmL^{-1} for JEV. The disposable nature of this biosensor strip will prevent cross-contamination from previous samples in clinical diagnosis.

The capability performance of this electrochemical immunosensor strips was demonstrated by using samples containing JEV positive human serum and Dengue Antigen. Results showed the sample containing JEV positive human serum, the R_{et} (1421 ohm) was observed to be substantially higher as compared to the R_{et} (1099 ohm) of BSA blocked/JEV antibody immobilized CNPs on SPCE strips. These results indicated that the CNPs modified SPCE electrochemical biosensor strip was capable of JEV detection in human serums and potential for clinical application.

The selectivity of this biosensor strip was proven as in the presence of Dengue antigen, the R_{et} (1073 ohm) was observed to have no significant changes as compared with R_{et} (1099 ohm) of BSA blocked/JEV antibody immobilized CNPs on SPCE strips. This is because Dengue antigen was unable to form antigen-antibody non-conductive layer with the antibody of JEV.

For further enhancement of the CNPs sensitivity prepared in Chapter 4, the novel hybrid gold-carbon nanoparticles (Au-CNPs) was successfully fabricated by using self-assembly methods in the mixture of gold nanoparticles (AuNPs) with chitosan CNPs. The

hybrid Au-CNPs that fabricated found can minimizes the limitations as well as providing additional synergistic properties, that is, higher effective surface area, catalytic activity, electrical conductivity, water solubility, and biocompatibility. As expected, this novel biosensor strips exhibited rapid response and results of analysis were obtainable below 10 minutes and has shown an extremely low LOD of 0.29 ngmL^{-1} and detection sensitivity were 0.04 ngmL^{-1} for JEV.

Generally, comparing the findings that reported in Chapter 4 (carbon-based nanoparticles electrochemical biosensor) and Chapter 5 (hybrid gold and carbon-based nanoparticles electrochemical biosensor), although results show that the hybrid Au-CNPs exhibited slightly better LOD and more sensitive toward the detection of JEV as compare with chitosan CNPs, however, when considering the high cost of AuNPs, CNPs modified SPCE electrochemical biosensor still is a better choice for the development of point-of-care, economical and fast device for detection of JEV. Thus, this can conclude that this studies had successfully demonstrated the potential application of CNPs prepared from chitosan nanoparticles as sensing probe for selective detection of JEV.

6.2 Recommendations for Future Works

The weaknesses and limitations of each experiment in this study have led to the following recommendations for future works. For Chapter 3, synthesis and characterization of various carbon-based nanoparticles (CNPs), result showed that the CNPs from different types of precursor by refluxing with acid resulting in various sizes of the CNPs formed. Based on the electrochemical characterisation measurement analyse by using CV and EIS, it can be explained that the smaller the size of the nanoparticles, the larger the total surface area, the higher electrical conductivity of the CNPs on the electrode surface. Thus,

recommended that repeat and further investigate more optimal parameters (refluxing temperature, concentration or amount of the reactants and refluxing time) on all the various types of CNPs to modify and fabricate a better, greater and high sensitivity, selectivity and capability of electrochemical immunosensor.

Recently, the severe outbreak of the coronavirus (temporary known as 2019-nCoV) in Wuhan city and the disease can easily spread around the world has already killed more than thousand of peoples. Therefore, it is crucial to have a portable and point-of-care device for the early detection of the disease. In conjunction with this, it is recommended that further modification or functionality to be added onto the CNPs for the fabrication of extraordinary biosensor. The addition will be able to create different voltage waveforms to run a range of electrochemical experiments for the detection for various type of the disease or virus.

Strongly recommended on the development of a module for a low-cost smartphone-based biosensor (smartphone-based electrochemical biosensor module). The module contains a low power potentiostat that interfaces and harvests power from a smartphone through the phone's audio jack. A prototype with two different potentiostat designs was constructed and used to conduct proof of concept cyclic voltammetry experiments with potassium ferro-/ferricyanide ($K_4[Fe(CN)_6]$ / $K_3[Fe(CN)_6]$) in a side-by-side comparison with a laboratory grade instrument. Such a device would enable numerous point-of-care (POC) applications allowing individuals to run simple medical tests without visiting a lab or doctor's office, thereby improving the convenience and speed of medical diagnoses.

REFERENCES

- Abo-Hamad, A., AlSaadi, M. A., Hayyan, M., Juneidi, I., & Hashim, M. A. (2016). Ionic liquid-carbon nanomaterial hybrids for electrochemical sensor applications: a review. *Electrochimica Acta*, 193, 321-343.
- Akhter, I. (2017). *Development of novel pH sensor based on nanoparticle modified electrochemical sensing platform* (Doctoral dissertation, Khulna University of Engineering & Technology (KUET), Khulna, Bangladesh).
- Alaneed, R., Golitsyn, Y., Hauenschild, T., Pietzsch, M., Reichert, D., & Kressler, J. (2021). Network formation by. *Polymer International*, 70(1), 135-144.
- Al-Jumaili, A., Alancherry, S., Bazaka, K., & Jacob, M. (2017). Review on the antimicrobial properties of carbon nanostructures. *Materials*, 10(9), 1009-1066.
- Ambrosi, A., Chua, C. K., Bonanni, A., & Pumera, M. (2014). Electrochemistry of graphene and related materials. *Chemical Reviews*, 114(14), 7150-7188.
- Amicizia, D., Zangrillo, F., Lai, P. L., Iovine, M., & Panatto, D. (2018). Overview of Japanese encephalitis disease and its prevention. Focus on IC51 vaccine (IXIARO®). *Journal of Preventive Medicine and Hygiene*, 59(1), 99-107.
- Angione, M. D., Pilolli, R., Cotrone, S., Magliulo, M., Mallardi, A., Palazzo, G., ... & Torsi, L. (2011). Carbon based materials for electronic bio-sensing. *Materials Today*, 14(9), 424-433.
- Anusha, J. R., Kim, H. J., Fleming, A. T., Das, S. J., Yu, K. H., Kim, B. C., & Raj, C. J. (2014). Simple fabrication of ZnO/Pt/chitosan electrode for enzymatic glucose biosensor. *Sensors and Actuators B: Chemical*, 202, 827-833.

- Arduini, F., Micheli, L., Moscone, D., Palleschi, G., Piermarini, S., Ricci, F., & Volpe, G. (2016). Electrochemical biosensors based on nanomodified screen-printed electrodes: Recent applications in clinical analysis. *TrAC Trends in Analytical Chemistry*, 79, 114-126.
- Asadian, E., Ghalkhani, M. and Shahrokhian, S. (2019). Electrochemical Sensing Based on Carbon Nanoparticles: A Review. *Sensors and Actuators B: Chemical*, 293, 183-209.
- Avadi, M.R., Sadeghi, A.M.M., Mohammadpour, N., Abedin, S., Atyabi, F., Dinarvand, R. & Rafiee-Tehrani, M. (2010). Preparation and characterization of insulin nanoparticles using chitosan and Arabic gum with ionic gelation method. *Nanomedicine: Nanotechnology, Biology and Medicine*, 6(1), 58-63.
- Ayyar, B. V., & Arora, S. (2013). Antibody-based biosensors for detection of veterinary viral pathogens. *Advance Animal Veterinary Science*, 1(4S), 37-44.
- Baca, J., Severns, V., Lovato, D., Branch, D., & Larson, R. (2015). Rapid detection of Ebola virus with a reagent-free, point-of-care biosensor. *Sensors*, 15(4), 8605-8614.
- Bacakova, L., Broz, A., Liskova, J., Stankova, L., Potocky, S., & Kromka, A. (2016). The application of nanodiamond in biotechnology and tissue engineering. *Diamond and Carbon Composites and Nanocomposites*, 3, 59-88.
- Bakker, E. and Telting-Diaz, M., 2002. Electrochemical sensors. *Analytical Chemistry*, 74(12), 2781-2800.
- Bayda, S., Hadla, M., Palazzolo, S., Kumar, V., Caligiuri, I., Ambrosi, E., & Riello, P. (2017). Bottom-up synthesis of carbon nanoparticles with higher doxorubicin efficacy. *Journal of Controlled Release*, 248, 144-152.

- Beitollahi, H., Safaei, M., & Tajik, S. (2019). Voltammetric and amperometric sensors for determination of epinephrine: A short review (2013-2017). *Journal of Electrochemical Science and Engineering*, 9(1), 27-43.
- Bharucha, T., Sengvilaipaseuth, O., Vongsouvath, M., Vongsouvath, M., Davong, V., Panyanouvong, P., & Dubot-Pérès, A. (2018). Development of an improved RT-qPCR Assay for detection of Japanese encephalitis virus (JEV) RNA including a systematic review and comprehensive comparison with published methods. *PloS ONE*, 13(3), 1-18.
- Bhatnagar, I., Mahato, K., Ealla, K. K. R., Asthana, A., & Chandra, P. (2018). Chitosan stabilized gold nanoparticle mediated self-assembled glip nanobiosensor for diagnosis of invasive aspergillosis. *International Journal of Biological Macromolecules*, 110, 449-456.
- Biegler, T., & Woods, R. (1973). The standard hydrogen electrode. A misrepresented concept. *Journal of Chemical Education*, 50(9), 604-605.
- Bonaparte, M., Dweik, B., Feroldi, E., Meric, C., Bouckenoghe, A., Hildreth, S., Hu, B., Yoksan, S. & Boaz, M. (2014). Immune response to live-attenuated Japanese encephalitis vaccine (JE-CV) neutralizes Japanese encephalitis virus isolates from South-East Asia and India. *BMC Infectious Diseases*, 14(1), 156-162.
- Borgmann, S., Schulte, A., Neugebauer, S., & Schuhmann, W. (2011). Amperometric biosensors. *Advances in Electrochemical Science and Engineering*, 2, 1-83.
- Brajter-Toth, A., & Chambers, J. (Eds.). (2002). *Electroanalytical Methods of Biological Materials*. CRC Press, New York: Marcel Dekker, Inc.
- Button, V. (2015). *Principles of Measurement and transduction of biomedical variables*. Academic Press, USA: Elsevier.

- Cao, L., Jia, J., & Wang, Z. (2008). Sensitive determination of Cd and Pb by differential pulse stripping voltammetry with in-situ bismuth-modified zeolite doped carbon paste electrodes. *Electrochimica Acta*, 53(5), 2177-2182.
- Cardosa, M.J., Wang, S.M., Sum, M.S.H. & Tio, P.H. (2002). Antibodies against prM protein distinguish between previous infection with dengue and Japanese encephalitis viruses. *BMC Microbiology*, 2(1), 9-15.
- Carter, M., & Shieh, J. C. (2015). *Guide to research techniques in neuroscience*. Academic Press. 2nd ed., USA: Elsevier.
- Cha, G. W., Cho, J. E., Ju, Y. R., Hong, Y. J., Han, M. G., Lee, W. J., & Jeong, Y. E. (2014). Comparison of four serological tests for detecting antibodies to Japanese encephalitis virus after vaccination in children. *Osong Public Health and Research Perspectives*, 5(5), 286-291.
- Chen, Y. J., Chen, M., Hsieh, Y. C., Su, Y. C., Wang, C. H., Cheng, C. M., & Chuang, K. H. (2018). Development of a highly sensitive enzyme-linked immunosorbent assay (ELISA) through use of poly-protein G-expressing cell-based microplates. *Scientific Reports*, 8(1), 1-11.
- Chin, S. F., Lim, L. S., Pang, S. C., Sum, M. S. H., & Perera, D. (2017). Carbon nanoparticle modified screen printed carbon electrode as a disposable electrochemical immunosensor strip for the detection of Japanese encephalitis virus. *Microchimica Acta*, 184(2), 491-497.
- Chin, S. F., Pang, S. C., & Tay, S. H. (2011). Size controlled synthesis of starch nanoparticles by a simple nanoprecipitation method. *Carbohydrate Polymers*, 86(4), 1817-1819.

- Christwardana, M., & Frattini, D. (2018). Electrochemical Study of Enzymatic Glucose Sensors Biocatalyst: Thermal Degradation after Long-Term Storage. *Chemosensors*, 6(4), 53-62.
- Coker, R.J., Hunter, B.M., Rudge, J.W., Liverani, M. & Hanvoravongchai, P. (2011). Emerging infectious diseases in southeast Asia: regional challenges to control. *The Lancet*, 377(9765), 599-609.
- Cui, H., Hong, C., Ying, A., Yang, X., & Ren, S. (2013). Ultrathin gold nanowire-functionalized carbon nanotubes for hybrid molecular sensing. *ACS Nano*, 7(9), 7805-7811.
- Cui, L., Li, Y., Lu, M., Tang, B., & Zhang, C. Y. (2018). An ultrasensitive electrochemical biosensor for polynucleotide kinase assay based on gold nanoparticle-mediated lambda exonuclease cleavage-induced signal amplification. *Biosensors and Bioelectronics*, 99, 1-7.
- Cui, R., Huang, H., Yin, Z., Gao, D., & Zhu, J. J. (2008a). Horseradish peroxidase-functionalized gold nanoparticle label for amplified immunoanalysis based on gold nanoparticles/carbon nanotubes hybrids modified biosensor. *Biosensors and Bioelectronics*, 23(11), 1666-1673.
- Cui, R., Liu, C., Shen, J., Gao, D., Zhu, J. J., & Chen, H. Y. (2008b). Gold nanoparticle–colloidal carbon nanosphere hybrid material: preparation, characterization, and application for an amplified electrochemical immunoassay. *Advanced Functional Materials*, 18(15), 2197-2204.
- Daraee, H., Eatemadi, A., Abbasi, E., Fekri Aval, S., Kouhi, M., & Akbarzadeh, A. (2016). Application of gold nanoparticles in biomedical and drug delivery. *Artificial Cells, Nanomedicine and Biotechnology*, 44(1), 410-422.

- Datta, S., Christena, L. R., & Rajaram, Y. R. S. (2013). Enzyme immobilization: an overview on techniques and support materials. *Biotechnology*, 3(1), 1-9.
- De Lima, F., Lucca, B. G., Barbosa, A. M., Ferreira, V. S., Moccelini, S. K., Franzoi, A. C., & Vieira, I. C. (2010). Biosensor based on pequi polyphenol oxidase immobilized on chitosan crosslinked with cyanuric chloride for thiodicarb determination. *Enzyme and Microbial Technology*, 47(4), 153-158.
- Dhull, V., Gahlaut, A., Dilbaghi, N., & Hooda, V. (2013). Acetylcholinesterase biosensors for electrochemical detection of organophosphorus compounds: a review. *Biochemistry Research International*, 2013, 1-18.
- Ding, F., Deng, H., Du, Y., Shi, X., & Wang, Q. (2014). Emerging chitin and chitosan nanofibrous materials for biomedical applications. *Nanoscale*, 6(16), 9477-9493.
- Dongol, M., El-Denglawey, A., Abd El Sadek, M. S., & Yahia, I. S. (2015). Thermal annealing effect on the structural and the optical properties of Nano CdTe films. *Optik*, 126(14), 1352-1357.
- Du, Y., Luo, X. L., Xu, J. J., & Chen, H. Y. (2007). A simple method to fabricate a chitosan-gold nanoparticles film and its application in glucose biosensor. *Bioelectrochemistry*, 70(2), 342-347.
- Erlanger, T. E., Weiss, S., Keiser, J., Utzinger, J., & Wiedenmayer, K. (2009). Past, present, and future of Japanese encephalitis. *Emerging Infectious Diseases*, 15(1), 1-7.
- Fajac, I., Lacronique, J., Lockhart, A., Dall'Ava-Santucci, J., & Dusser, D. J. (1998). Silver/silver chloride electrodes for measurement of potential difference in human bronchi. *Thorax*, 53(10), 879-881.

- Fan, W., Yan, W., Xu, Z. and Ni, H., 2012. Formation mechanism of monodisperse, low molecular weight chitosan nanoparticles by ionic gelation technique. *Colloids and Surfaces B: Biointerfaces*, 90, 21-27.
- Fang, Y., & Wang, E. (2013). Electrochemical biosensors on platforms of graphene. *Chemical Communications*, 49(83), 9526-9539.
- Feng, W., Liang, C., Gong, H., & Cai, C. (2018). Sensitive detection of Japanese encephalitis virus by surface molecularly imprinted technique based on fluorescent method. *New Journal of Chemistry*, 42(5), 3503-3508.
- Ferri, S., Kojima, K., & Sode, K. (2011). Review of glucose oxidases and glucose dehydrogenases: a bird's eye view of glucose sensing enzymes. *Journal of Diabetes Science and Technology*, 5(5), 1068-1076.
- Filgueira, L., & Lannes, N. (2019). Review of Emerging Japanese Encephalitis Virus: New Aspects and Concepts about Entry into the Brain and Inter-Cellular Spreading. *Pathogens*, 8(3), 111-130.
- Gaminian, H., Montazer, M., Bahi, A., Karaaslan, M., & Ko, F. (2019). Capacitance performance boost of cellulose-derived carbon nanofibers via carbon and silver nanoparticles. *Cellulose*, 26(4), 2499-2512.
- Gayen, M., Gupta, P., Morazzani, E. M., Gaidamakova, E. K., Knollmann-Ritschel, B., Daly, M. J., & Maheshwari, R. K. (2017). *Deinococcus Mn²⁺-peptide complex: A novel approach to alphavirus vaccine development. Vaccine*, 35(29), 3672-3681.
- Geng, X., Zhang, F., Gao, Q., & Lei, Y. (2016). Sensitive Impedimetric Immunoassay of Japanese Encephalitis Virus Based on Enzyme Biocatalyzed Precipitation on a Gold Nanoparticle-modified Screen-printed Carbon Electrode. *Analytical Sciences*, 32(10), 1105-1109.

- Gomes, L. P., Paschoalin, V. M. F., & Del Aguila, E. M. (2017). Chitosan nanoparticles: Production, physicochemical characteristics and nutraceutical applications. *Revista Virtual de Quimica*, 5(5), 1068-1076.
- Grenha, A. (2012). Chitosan nanoparticles: a survey of preparation methods. *Journal of drug Targeting*, 20(4), 291-300.
- Grzybowski, A. K. (1958). The Standard Potential of the Calomel Electrode and its Application in Accurate Physicochemical Measurements and The Standard Potential. *The Journal of Physical Chemistry*, 62(5), 550-555.
- Guo, J., Jia, X., Liu, Y., Wang, S., Cao, J., Zhang, B., & Wang, W. (2020). Screening of Natural Extracts for Inhibitors against Japanese Encephalitis Virus Infection. *Antimicrobial Agents and Chemotherapy*, 64(3), 1-11.
- Guo, M., Fang, H., Wang, R., Yang, Z., & Xu, X. (2011). Electrodeposition of chitosan-glucose oxidase biocomposite onto Pt–Pb nanoparticles modified stainless steel needle electrode for amperometric glucose biosensor. *Materials in Medicine*, 22(8), 85-92.
- Gupta, B. P., Mishra, S. K., Manandhar, K. D., Malla, R., Tamarakar, C. S., Bajarcharya, A., & Pokhrel, S. (2014). Comparison of CPE NT and PRNT assays for estimating Neutralizing antibody titres against Japanese Encephalitis Virus. *International Journal Current Microbiology Applied Science*, 3(1), 407-413.
- Hajihosseini, S., Nasirizadeh, N., Hejazi, M. S., & Yaghmaei, P. (2016). A sensitive DNA biosensor fabricated from gold nanoparticles and graphene oxide on a glassy carbon electrode. *Materials Science and Engineering: C*, 61, 506-515.
- Hayat, A., & Marty, J. (2014). Disposable screen-printed electrochemical sensors: Tools for environmental monitoring. *Sensors*, 14(6), 10432-10453.

- Heffelfinger, J. D., Li, X., Batmunkh, N., Grabovac, V., Diorditsa, S., Liyanage, J. B., & Chu, S. Y. (2017). Japanese encephalitis surveillance and immunization—Asia and Western Pacific Regions, 2016. *MMWR. Morbidity and Mortality Weekly Report*, 66(22), 579-583.
- Hobson-Peters, J. (2012). Approaches for the development of rapid serological assays for surveillance and diagnosis of infections caused by zoonotic flaviviruses of the Japanese encephalitis virus serocomplex. *BioMed Research International*, 2012, 1-15.
- Hombach, J., Solomon, T., Kurane, I., Jacobson, J., & Wood, D. (2005). Report on a WHO consultation on immunological endpoints for evaluation of new Japanese encephalitis vaccines, WHO, Geneva, 2–3 September, 2004. *Vaccine*, 23(45), 5205-5211.
- Huang, K.J., Li, J., Liu, Y.M., Cao, X., Yu, S., & Yu, M. (2012a). Disposable immunoassay for hepatitis B surface antigen based on a graphene paste electrode functionalized with gold nanoparticles and a Nafion-cysteine conjugate. *Microchimica Acta*, 177(3-4), 419-426.
- Huang, X., Qi, X., Boey, F., & Zhang, H. (2012b). Graphene-based composites. *Chemical Society Reviews*, 41(2), 666-686.
- Huy, T. Q., Hanh, N. T. H., Thuy, N. T., Van Chung, P., Nga, P. T., & Tuan, M. A. (2011). A novel biosensor based on serum antibody immobilization for rapid detection of viral antigens. *Talanta*, 86, 271-277.
- Hyun, W. J., Park, O. O., & Chin, B. D. (2013). Foldable graphene electronic circuits based on paper substrates. *Advanced Materials*, 25(34), 4729-4734.
- Ibrahim, H. M., El-Bisi, M. K., Taha, G. M., & El-Alfy, E. A. (2015). Chitosan nanoparticles loaded antibiotics as drug delivery biomaterial. *Journal Applied Pharmacy Science*, 5(10), 85-90.

- Inagaki, M., Kang, F., Toyoda, M., & Konno, H. (2013). *Advanced Materials Science and Engineering of Carbon*, USA: Butterworth-Heinemann.
- Jazayeri, M. H., Amani, H., Pourfatollah, A. A., Pazoki-Toroudi, H., & Sedighimoghaddam, B. (2016). Various methods of gold nanoparticles (GNPs) conjugation to antibodies. *Sensing and Bio-sensing Research*, 9, 17-22.
- Jirakittidul, K., Vittayakorn, N., Manrean, R., Pornteeranawapat, N., & Neamyoooyong, S. (2019). Acid modified multiwalled carbon nanotubes condition by reflux. *Materials Research Express*, 6(11), 115003-115008.
- Johnson, B. W., Kosoy, O., Hunsperger, E., Beltran, M., Delorey, M., Guirakhoo, F., & Monath, T. (2009). Evaluation of chimeric Japanese encephalitis and dengue viruses for use in diagnostic plaque reduction neutralization tests. *Clinical Vaccine Immunology*, 16(7), 1052-1059.
- Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L. & Daszak, P., (2008). Global trends in emerging infectious diseases. *Nature*, 451(7181), 990-999.
- JoVE Science Education Database, (2019). *Material Engineering: Electrochemical Impedance Spectroscopy* [online] Available at: <https://www.jove.com/science-education/10491/electrochemical-impedance-spectroscopy> [Assessed on 6 February 2020]
- Kai, H., Kato, Y., Toyosato, R., & Nishizawa, M. (2018). Fluid-permeable enzymatic lactate sensors for micro-volume specimen. *Analyst*, 143(22), 5545-5551.
- Khalil, I., Julkapli, N. M., Yehye, W. A., Basirun, W. J., & Bhargava, S. K. (2016). Graphene–gold nanoparticles hybrid—synthesis, functionalization, and application in a electrochemical and surface-enhanced raman scattering biosensor. *Materials*, 9(6), 406-412.

- Khan, I., Saeed, K., & Khan, I. (2019). Nanoparticles: Properties, applications and toxicities. *Arabian Journal of Chemistry*, 12(7), 908-931.
- Khodashenas, B., & Ghorbani, H. R. (2019). Synthesis of silver nanoparticles with different shapes. *Arabian Journal of Chemistry*, 12(8), 1823-1838.
- Kokorina, A. A., Sapelkin, A. V., Sukhorukov, G. B., & Goryacheva, I. Y. (2019). Luminescent carbon nanoparticles separation and purification. *Advances in Colloid and Interface Science*, 102043, 1-37.
- Kumar, K., Arshad, S. S., Selvarajah, G. T., Abu, J., Toung, O. P., Abba, Y., ... & Ong, B. L. (2018). Japanese encephalitis in Malaysia: An overview and timeline. *Acta Tropica*, 185, 219-229.
- Lai, H. C., Chin, S. F., Pang, S. C., Sum, H., Sia, M., & Perera, D. (2017). Carbon nanoparticles based electrochemical biosensor strip for detection of Japanese Encephalitis Virus. *Journal of Nanomaterials*, 2017, 1-7.
- Lannes, N., Neuhaus, V., Scolari, B., Kharoubi-Hess, S., Walch, M., Summerfield, A., & Filgueira, L. (2017). Interactions of human microglia cells with Japanese encephalitis virus. *Virology Journal*, 14(1), 8.
- Lauter, V., Lauter, H., Glavic, A., & Toperverg, B. (2016). Reference module in materials science and materials engineering. *Physica B: Condensed Matter*, 241, 1092-1094.
- Li, F., Mei, L., Li, Y., Zhao, K., Chen, H., Wu, P., Hu, Y. & Cao, S., 2011. Facile fabrication of magnetic gold electrode for magnetic beads-based electrochemical immunoassay: Application to the diagnosis of Japanese encephalitis virus. *Biosensors and Bioelectronics*, 26(10), 4253-4256.

- Li, X., Chang, J., Xu, F., Wang, X., Lang, Y., Gao, Z., & Jiang, K. (2015). Pyrolytic synthesis of carbon quantum dots, and their photoluminescence properties. *Research on Chemical Intermediates*, 41(2), 813-819.
- Li, Y., Hsieh, C. H., Lai, C. W., Chang, Y. F., Chan, H. Y., Tsai, C. F., & Wu, L. C. (2017). Tyramine detection using PEDOT: PSS/AuNPs/1-methyl-4-mercaptopyridine modified screen-printed carbon electrode with molecularly imprinted polymer solid phase extraction. *Biosensors and Bioelectronics*, 87, 142-149.
- Liddell, J. E. (2001a). Enzyme-linked Immunosorbent Assay (ELISA) to Measure Pure Protein. e LS.
- Liddell, J. E. (2001b). Production of Monoclonal Antibodies. e LS.
- Lin, L., Xue, L., Duraiarasan, S., & Haiying, C. (2018). Preparation of ϵ -polylysine/chitosan nanofibers for food packaging against Salmonella on chicken. *Food Packaging and Shelf Life*, 17, 134-141.
- Lindsey, N. P., Staples, J. E., Jones, J. F., Sejvar, J. J., Griggs, A., Iskander, J., & Fischer, M. (2010). Adverse event reports following Japanese encephalitis vaccination in the United States, 1999–2009. *Vaccine*, 29(1), 58-64.
- Liu, C., Xu, C., Xue, N., Sun, J. H., Cai, H., Li, T., & Wang, J. (2018). Enzyme Biosensors for Point-of-Care Testing. *MEMS Sensors: Design and Application*, 73249, 49-70.
- Liu, S., & Guo, X. (2012). Carbon nanomaterials field-effect-transistor-based biosensors. *NPG Asia Materials*, 4(8), 23-30.
- Liu, W., Diao, H., Chang, H., Wang, H., Li, T., & Wei, W. (2017). Green synthesis of carbon dots from rose-heart radish and application for Fe^{3+} detection and cell imaging. *Sensor Actuator B: Chemical*, 241, 190-198.

- Liu, X., Pang, J., Xu, F., & Zhang, X. (2016). Simple approach to synthesize amino-functionalized carbon dots by carbonization of chitosan. *Scientific Reports*, 6, 31-40.
- Lu, W., Qin, X., Liu, S., Chang, G., Zhang, Y., Luo, Y., Asiri, A.M., Al-Youbi, A.O. & Sun, X. (2012). Economical, green synthesis of fluorescent carbon nanoparticles and their use as probes for sensitive and selective detection of mercury (II) ions. *Analytical Chemistry*, 84(12), 5351-5357.
- Lv, X., Geng, Z., Su, Y., Fan, Z., Wang, S., Fang, W., & Chen, H. (2019). Label-Free Exosome Detection Based on a Low-Cost Plasmonic Biosensor Array Integrated with Microfluidics. *Langmuir*, 35(30), 9816-9824.
- Macdonald, J. R., & Johnson, W. B. (2018). Fundamentals of impedance spectroscopy. *Impedance Spectroscopy: Theory, Experiment, and Applications*, 1-20.
- Maduraiveeran, G., & Jin, W. (2017). Nanomaterials based electrochemical sensor and biosensor platforms for environmental applications. *Trends in Environmental Analytical Chemistry*, 13, 10-23.
- Malhotra, S., Verma, A., Tyagi, N., & Kumar, V. (2017). Biosensors: Principle, types and applications. *International Journal of Advance Research and Innovative Ideas of Education*, 3(2), 3639-3644.
- Mansfield, K. L., Hernandez-Triana, L. M., Banyard, A. C., Fooks, A. R., & Johnson, N. (2017). Japanese encephalitis virus infection, diagnosis and control in domestic animals. *Veterinary Microbiology*, 201, 85-92.
- Masotti, A., Marino, F., Ortaggi, G., & Palocci, C. (2007). Fluorescence and scanning electron microscopy of chitosan/DNA nanoparticles for biological applications. *Modern Research and Educational Topics in Microscopy*, 2, 690-696.

- Matsui, T., Kitagawa, Y., Okumura, M., & Shigeta, Y. (2015). Accurate standard hydrogen electrode potential and applications to the redox potentials of vitamin C and NAD/NADH. *The Journal of Physical Chemistry A*, 119(2), 369-376.
- Meenu, M., & Xu, B. (2019). A critical review on anti-diabetic and anti-obesity effects of dietary resistant starch. *Critical Reviews in Food Science and Nutrition*, 59(18), 3019-3031.
- Mei, L., Wu, P., Ye, J., Gao, G., Shao, L., Huang, S., & Cao, S. (2012). Development and application of an antigen capture ELISA assay for diagnosis of Japanese encephalitis virus in swine, human and mosquito. *Virology Journal*, 9(1), 4-14.
- Mendes, R., Wróbel, P., Bachmatiuk, A., Sun, J., Gemming, T., Liu, Z., & Rummeli, M. (2018). Carbon Nanostructures as a Multi-Functional Platform for Sensing Applications. *Chemosensors*, 6(4), 60-71.
- Merkoçi, A. (2010). Nanoparticles-based strategies for DNA, protein and cell sensors. *Biosensors and Bioelectronics*, 26(4), 1164-1177.
- Messaoud, N. B., Ghica, M. E., Dridi, C., Ali, M. B., & Brett, C. M. (2017). Electrochemical sensor based on multiwalled carbon nanotube and gold nanoparticle modified electrode for the sensitive detection of bisphenol A. *Sensors and Actuators B: Chemical*, 253, 513-522.
- Metters, J. P., Kadara, R. O., & Banks, C. E. (2011). New directions in screen printed electroanalytical sensors: an overview of recent developments. *Analyst*, 136(6), 1067-1076.
- Mindtouch®, (2019). *LibreTexts: Chemistry* [online] Available at: [https://chem.libretexts.org/Bookshelves/Physical_and_Theoretical_Chemistry_Textbook_Maps/Book%3A_Thermodynamics_and_Chemical_Equilibrium_\(Ellgen\)/17%3A](https://chem.libretexts.org/Bookshelves/Physical_and_Theoretical_Chemistry_Textbook_Maps/Book%3A_Thermodynamics_and_Chemical_Equilibrium_(Ellgen)/17%3A)

_Electrochemistry/17.10%3A_The_Standard_Hydrogen_Electrode_(S.H.E) [Assessed on 6 February 2020]

Mishra, R., & Militky, J. (2018). *Nanotechnology in Textiles: Theory and Application*, UK: Woodhead Publishing.

Mohamed, E. F. (2017). Nanotechnology: future of environmental air pollution control. *Environmental Management and Sustainable Development*, 6(2), 429-454.

Moharana, S., Kar, S. K., Mishra, M. K., & Mahaling, R. N. (2019). Synthesis and Properties of Graphene and Graphene Oxide-Based Polymer Composites. In: Sahoo S., Tiwari S., Nayak G. (Eds.) *Surface Engineering of Graphene. Carbon Nanostructures* (175-201). UK: Springer, Chamn.

Momenpoor, B., Danafar, F., & Bakhtiari, F. (2019). Size Controlled Preparation of Starch Nanoparticles from Wheat Through Precipitation at Low Temperature. *Journal of Nano Research*, 56, 131-141.

Morley, P. S., & Pusterla, N. (2013). Viral respiratory disease in athletic horses. *Equine Sports Medicine and Surgery*, 2nd ed., 649-664. UK: Elsevier Ltd.

Muniandy, S., Teh, S. J., Thong, K. L., Thiha, A., Dinshaw, I. J., Lai, C. W., ... & Leo, B. F. (2019). Carbon nanomaterial-based electrochemical biosensors for foodborne bacterial detection. *Critical Reviews in Analytical Chemistry*, 49(6), 510-533.

Murugaiyan, S. B., Ramasamy, R., Gopal, N., & Kuzhandaivelu, V. (2014). Biosensors in clinical chemistry: An overview. *Advanced Biomedical Research*, 3, 67-80.

Muthusankar, E., & Ragupathy, D. (2018). Chitosan Based Nanocomposite Biosensors: A Recent Review. *Sensor Letters*, 16(2), 81-91.

Nain, M., Abdin, M. Z., Kalia, M., & Vрати, S. (2016). Japanese encephalitis virus invasion of cell: allies and alleys. *Reviews in Medical Virology*, 26(2), 129-141.

- Nguyen, H., Lee, S., Lee, U., Fermin, C., & Kim, M. (2019). Immobilized enzymes in biosensor applications. *Materials*, 12(1), 121-129.
- Niu, P., Fernández-Sánchez, C., Gich, M., Navarro-Hernández, C., Fanjul-Bolado, P., & Roig, A. (2016). Screen-printed electrodes made of a bismuth nanoparticle porous carbon nanocomposite applied to the determination of heavy metal ions. *Microchimica Acta*, 183(2), 617-623.
- O'connell, M. J. (2018). *Carbon nanotubes: Properties and Applications*. 1st ed., Boca Raton: CRC press.
- Oh, S. I., Kim, J. C., & Kim, D. W. (2019). Cellulose-derived tin-oxide-nanoparticle-embedded carbon fibers as binder-free flexible Li-ion battery anodes. *Cellulose*, 26(4), 2557-2571.
- Ojani, R., Alinezhad, A., & Abedi, Z. (2013). A highly sensitive electrochemical sensor for simultaneous detection of uric acid, xanthine and hypoxanthine based on poly (l-methionine) modified glassy carbon electrode. *Sensors and Actuators B: Chemical*, 188, 621-630.
- Oliver, N. S., Toumazou, C., Cass, A. E. G., & Johnston, D. G. (2009). Glucose sensors: a review of current and emerging technology. *Diabetic Medicine*, 26(3), 197-210.
- Othman, N., Masarudin, M., Kuen, C., Dasuan, N., & Abdullah, L. (2018). Synthesis and Optimization of Chitosan Nanoparticles Loaded with L-Ascorbic Acid and Thymoquinone. *Nanomaterials*, 8(11), 920-930.
- Pan, D., Gu, Y., Lan, H., Sun, Y., & Gao, H. (2015). Functional graphene-gold nanocomposite fabricated electrochemical biosensor for direct and rapid detection of bisphenol A. *Analytica Chimica Acta*, 853, 297-302.

- Pandiaraj, M., Sethy, N. K., Bhargava, K., Kameswararao, V., & Karunakaran, C. (2014). Designing label-free electrochemical immunosensors for cytochrome c using nanocomposites functionalized screen-printed electrodes. *Biosensors and Bioelectronics*, 54, 115-121.
- Pauliukaite, R., Ghica, M. E., Fatibello-Filho, O., & Brett, C. M. (2010). Electrochemical impedance studies of chitosan-modified electrodes for application in electrochemical sensors and biosensors. *Electrochimica Acta*, 55(21), 6239-6247.
- Peigney, A., Laurent, C., Flahaut, E., Bacsá, R. R., & Rousset, A. (2001). Specific surface area of carbon nanotubes and bundles of carbon nanotubes. *Carbon*, 39(4), 507-514.
- Pejcic, B., De Marco, R. & Parkinson, G. (2006). The role of biosensors in the detection of emerging infectious diseases. *Analyst*, 131(10), 1079-1090.
- Petty, M. C. (2008). Molecular electronics: from principles to practice. *John Wiley & Sons*.
- Piermarini, S., Migliorelli, D., Volpe, G., Massoud, R., Pierantozzi, A., Cortese, C., & Palleschi, G. (2013). Uricase biosensor based on a screen printed electrode modified with Prussian blue for detection of uric acid in human blood serum. *Sensors and Actuators, B. Chemical*, 179, 170–174
- Pilehvar, S., & De Wael, K. (2015). Recent advances in electrochemical biosensors based on fullerene-C60 nano-structured platforms. *Biosensors*, 5(4), 712-735.
- Poonsiri, T., Wright, G. S., Diamond, M. S., Turtle, L., Solomon, T., & Antonyuk, S. V. (2018). Structural study of the C-terminal domain of nonstructural protein 1 from Japanese encephalitis virus. *Journal of Virology*, 92(7), 1868-1877.
- Pumera, M., Ambrosi, A., Bonanni, A., Chng, E. L. K., & Poh, H. L. (2010). Graphene for electrochemical sensing and biosensing. *TrAC Trends in Analytical Chemistry*, 29(9), 954-965.

- Raj, C. R., & Ohsaka, T. (2002). Analytical applications of functionalized self-assembled monolayers on gold electrode: voltammetric sensing of DOPAC at the physiological level. *Electroanalysis: An International Journal Devoted to Fundamental and Practical Aspects of Electroanalysis*, 14(10), 679-684.
- Rao, C. N. R., & Seshadri, R. (2019). Fullerenes, Nanotubes, and Related Materials. In *Organic Molecular Solids: Properties and Applications*, (Jones, W., 1st ed.), UK: CRC Press.
- Rao, R. N., & Albaser, S. S. (2018). Nanomaterials in Chromatographic Sample Preparations. In *Nanomaterials in Chromatography*, 201-231. UK: Elsevier.
- Rassaei, L., Olthuis, W., Tsujimura, S., Sudhölter, E. J., & Van Den Berg, A. (2014). Lactate biosensors: current status and outlook. *Analytical and Bioanalytical Chemistry*, 406(1), 123-137.
- Ravalli, A., Dos Santos, G. P., Ferroni, M., Faglia, G., Yamanaka, H., & Marrazza, G. (2013). New label free CA125 detection based on gold nanostructured screen-printed electrode. *Sensors and Actuators B: Chemical*, 179, 194-200.
- Ravi. (2019). What are Biosensors? Principle, Working and Types. [online] Available at: <https://www.electronicshub.org/types-of-biosensors/> [Assessed on 6 February 2020].
- Rezaei, B., Ghani, M., Shoushtari, A. M., & Rabiee, M. (2016). Electrochemical biosensors based on nanofibres for cardiac biomarker detection: a comprehensive review. *Biosensors and Bioelectronics*, 78, 513-523.
- Rezaei, B., Saghebdoost, M., Sorkhe, A.M., & Majidi, N. (2011). Generation of a doxorubicin immunosensor based on a specific monoclonal antibody-nanogold-modified electrode. *Electrochimica Acta*, 56(16), 5702-5706.

- Robinson, J. S., Featherstone, D., Vasanthapuram, R., Biggerstaff, B. J., Desai, A., Ramamurty, N., & Johnson, B. W. (2010). Evaluation of three commercially available Japanese encephalitis virus IgM enzyme-linked immunosorbent assays. *The American Journal of Tropical Medicine and Hygiene*, 83(5), 1146-1155.
- Roy, S., Soin, N., Bajpai, R., Misra, D.S., McLaughlin, J.A., & Roy, S.S. (2011). Graphene oxide for electrochemical sensing applications. *Journal of Materials Chemistry*, 21(38), 14725-14731.
- Saito, N., Haniu, H., Usui, Y., Aoki, K., Hara, K., Takanashi, S., & Nomura, H. (2014). Safe clinical use of carbon nanotubes as innovative biomaterials. *Chemical Reviews*, 114(11), 6040-6079.
- Saylan, Y., Erdem, Ö., Ünal, S., & Denizli, A. (2019). An alternative medical diagnosis method: Biosensors for virus detection. *Biosensors*, 9(2), 65-74.
- Schuller, E., Klingler, A., Dubischar-Kastner, K., Dewasthaly, S., & Müller, Z. (2011). Safety profile of the Vero cell-derived Japanese encephalitis virus (JEV) vaccine IXIARO®. *Vaccine*, 29(47), 8669-8676.
- Schwartz-Duval, A. S., Mistry, N., Srivastava, I., Singh, J., Golk, K., & Pan, D. (2018). Facile Size-Controlled Synthesis of Fluorescent Carbon Nanoparticles with Size-Independent Optical Properties. *The Illinois Digital Environment for Access to Learning and Scholarship*, 2142, 1-17.
- Sedira, S., & Mendaci, B. (2020). Hydrothermal synthesis of spherical carbon nanoparticles (CNPs) for supercapacitor electrodes uses. *Materials for Renewable and Sustainable Energy*, 9(1), 1-18.

- Şenel, M. (2015). Simple method for preparing glucose biosensor based on in-situ polypyrrole cross-linked chitosan/glucose oxidase/gold bionanocomposite film. *Materials Science and Engineering: C*, 48, 287-293.
- Shafiei, M., Jafarizadeh-Malmiri, H., & Rezaei, M. (2019). Biological activities of chitosan and prepared chitosan-tripolyphosphate nanoparticles using ionic gelation method against various pathogenic bacteria and fungi strains. *Biologia*, 74(11), 1561-1568.
- Shang, H., Lu, Y., Zhao, F., Chao, C., Zhang, B., & Zhang, H. (2015). Preparing high surface area porous carbon from biomass by carbonization in a molten salt medium. *RSC Advances*, 5(92), 75728-75734.
- Shao, Y., Wang, J., Wu, H., Liu, J., Aksay, I. A., & Lin, Y., (2010). Graphene based electrochemical sensors and biosensors: a review. *Electroanalysis: An International Journal Devoted to Fundamental and Practical Aspects of Electroanalysis*, 22(10), 1027-1036.
- Sharma, A., Matharu, Z., Sumana, G., Solanki, P.R., Kim, C.G., & Malhotra, B.D., 2010. Antibody immobilized cysteamine functionalized-gold nanoparticles for aflatoxin detection. *Thin Solid Films*, 519(3), 1213-1218.
- Sharma, D., Kanchi, S., & Bisetty, K. (2019a). Biogenic synthesis of nanoparticles: A review. *Arabian Journal of Chemistry*, 12(8), 3576-3600.
- Sharma, G., Gupta, V. K., Agarwal, S., Kumar, A., Thakur, S., & Pathania, D. (2016). Fabrication and characterization of Fe@ MoPO nanoparticles: ion exchange behavior and photocatalytic activity against malachite green. *Journal of Molecular Liquids*, 219, 1137-1143.
- Sharma, G., Kumar, A., Sharma, S., Naushad, M., Dwivedi, R. P., Al-Othman, Z. A., & Mola, G. T. (2019b). Novel development of nanoparticles to bimetallic nanoparticles

- and their composites: a review. *Journal of King Saud University-Science*, 31(2), 257-269.
- Sheldon, R. A., & van Pelt, S. (2013). Enzyme immobilisation in biocatalysis: why, what and how. *Chemical Society Reviews*, 42(15), 6223-6235.
- Shen, C., Sun, Y., Wang, J., & Lu, Y. (2014). Facile route to highly photoluminescent carbon nanodots for ion detection, pH sensors and bioimaging. *Nanoscale*, 6(15), 9139–9147.
- Shering, M. A., Kannan, C., Kumar, K. S., Kumar, V. S., & Suganeshwari, M. (2011). Formulation of 5-fluorouracil loaded chitosan nanoparticles by emulsion droplet coalescence method for cancer therapy. *International Journal Pharmacy and Biological Architecture*, 2, 926-931.
- Shukla, S. K., & Mishra, A. K. (2014). Amorphous polymer-based nanocomposites for biosensor applications. *UJ Nanomaterials Science Research Group, University of Johannesburg, Africa do Sul*.
- Siangproh, W., Dungchai, W., Rattanarat, P., & Chailapakul, O. (2011). Nanoparticle-based electrochemical detection in conventional and miniaturized systems and their bioanalytical applications: A review. *Analytica Chimica Acta*, 690(1), 10-25.
- Solanki, P. R., Kaushik, A., Agrawal, V. V., & Malhotra, B. D. (2011). Nanostructured metal oxide-based biosensors. *NPG Asia Materials*, 3(1), 17-29.
- Soloducho, J., & Cabaj, J. (2013). Electrochemical nanosized biosensors: Perspectives and future of biocatalysts. *Journal of Analytical & Bioanalytical Techniques*, 5(7), 1-19.
- Solomon, T., Dung, N. M., Kneen, R., Nisalak, A., Vaughn, D. W., Farrar, J., & Cardoso, M. J. (1998). Rapid diagnosis of Japanese encephalitis by using an immunoglobulin M dot enzyme immunoassay. *Journal of Clinical Microbiology*, 36(7), 2030-2034.

- Song, Y., Zhu, S., Zhang, S., Fu, Y., Wang, L., Zhao, X., & Yang, B. (2015). Investigation from chemical structure to photoluminescent mechanism: a type of carbon dots from the pyrolysis of citric acid and an amine. *Journal of Materials Chemistry C*, 3(23), 5976–5984.
- Suginta, W., Khunkaewla, P., & Schulte, A. (2013). Electrochemical biosensor applications of polysaccharides chitin and chitosan. *Chemical Reviews*, 113(7), 5458-5479.
- Sum, M.S.H., Andrew, A., & Maling, M.A. (2015). Expression of recombinant E1 Glycoprotein of Chikungunya virus in Baculovirus expression system. *Journal of Biochemistry, Microbiology and Biotechnology*, 3(1), 26-29.
- Sumiyoshi, H., Mori, C., Fuke, I., Morita, K., Kuhara, S., Kondou, J., & Igarashi, A. (1987). Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. *Virology*, 161(2), 497-510.
- Sun, Y.P., Zhou, B., Lin, Y., Wang, W., Fernando, K.S., Pathak, P., Mezziani, M.J., Harruff, B.A., Wang, X., Wang, H., & Luo, P.G. (2006). Quantum-sized carbon dots for bright and colorful photoluminescence. *Journal of the American Chemical Society*, 128(24), 7756-7757.
- Supriya, S., Sriram, G., Ngaini, Z., Kavitha, C., Kurkuri, M., De Padova, I. P., & Hegde, G. (2019). The Role of Temperature on Physical–Chemical Properties of Green Synthesized Porous Carbon Nanoparticles. *Waste and Biomass Valorization*, 2019, 1-11.
- Suthanthiraraj, P. P. A., & Sen, A. K. (2019). Localized surface plasmon resonance (LSPR) biosensor based on thermally annealed silver nanostructures with on-chip blood-plasma separation for the detection of dengue non-structural protein NS1 antigen. *Biosensors and Bioelectronics*, 132, 38-46.

- Sy Kraft. (2018). What's to know about Japanese encephalitis? [Online] available at <https://www.medicalnewstoday.com/articles/181418.php> [Assessed on 6 February 2020]
- Taleat, Z., Khoshroo, A., & Mazloun-Ardakani, M. (2014). Screen-printed electrodes for biosensing: a review (2008–2013). *Microchimica Acta*, 181(9-10), 865-891.
- Tan, S.C., Chin, S.F., & Pang, S.C. (2017). Disposable carbon dots modified screen printed carbon electrode electrochemical sensor strip for selective detection of ferric ions. *Journal of Sensors*, 2017, 1-7.
- Tang, J., Su, B., Tang, D., & Chen, G. (2010). Conductive carbon nanoparticles-based electrochemical immunosensor with enhanced sensitivity for α -fetoprotein using irregular-shaped gold nanoparticles-labeled enzyme-linked antibodies as signal improvement. *Biosensors and Bioelectronics*, 25(12), 2657-2662.
- Tergestina, M. M., Sam, D. R. K., Prabhu, A. S., & Raj, J. P. (2018). Study on Japanese encephalitis in a tertiary care centre. *Medical Journal of Dr. DY Patil Vidyapeeth*, 11(2), 121-134.
- Theerthagiri, J., Salla, S., Senthil, R. A., Nithyadharseni, P., Madankumar, A., Arunachalam, P., & Kim, H. S. (2019). A review on ZnO nanostructured materials: energy, environmental and biological applications. *Nanotechnology*, 30(39), 392001-392018.
- Thévenot, D. R., Toth, K., Durst, R. A., & Wilson, G. S. (2001). Electrochemical biosensors: recommended definitions and classification. *Analytical Letters*, 34(5), 635-659.
- Tiwari, J. N., Vij, V., Kemp, K. C., & Kim, K. S. (2016). Engineered carbon-nanomaterial-based electrochemical sensors for biomolecules. *ACS Nano*, 10(1), 46-80.
- Tomassetti, M., Martini, E., Campanella, L., Favero, G., Carlucci, L., & Mazzei, F. (2013). Comparison of three immunosensor methods (surface plasmon resonance, screen-

- printed and classical amperometric immunosensors) for immunoglobulin G determination in human serum and animal or powdered milks. *Journal of Pharmaceutical and Biomedical Analysis*, 73, 90-98.
- Toumey, C. P. (2008). Reading Feynman into nanotechnology: A text for a new science. *Techné: Research in Philosophy and Technology*, 12(3), 133-168.
- Tran, Q.H., Mai, A.T., Nguyen, T.T., & Nguyen, T.H.H., 2012b. Towards the use of protein A-tagged gold nanoparticles for signal amplification of electrochemical immunosensors in virus detection. *Advances in Natural Sciences: Nanoscience and Nanotechnology*, 3(2), 025013.
- Tran, Q.H., Nguyen, T.H.H., Mai, A.T., Nguyen, T.T., Vu, Q.K., & Phan, T.N., 2012a. Development of electrochemical immunosensors based on different serum antibody immobilization methods for detection of Japanese encephalitis virus. *Advances in Natural Sciences: Nanoscience and Nanotechnology*, 3(1), 015012.
- Tuan, C. V., Huy, T. Q., Hieu, N. V., Tuan, M. A., & Trung, T. (2013). Polyaniline nanowires-based electrochemical immunosensor for label free detection of Japanese encephalitis virus. *Analytical Letters*, 46(8), 1229-1240.
- Tung, Y. T., Liang, J. J., Feng, H. T., Jhang, R. N., Hsieh, S. L., Lin, Y. L., & Wang, G. J. (2019). Investigation of the extremely weak interaction between the Japanese encephalitis virus and CLEC5A using a multivalent-interaction-enhancement sensing electrode. *Biosensors and Bioelectronics: X*, 2, 100024.
- Unser, S., Bruzas, I., He, J., & Sagle, L. (2015). Localized surface plasmon resonance biosensing: current challenges and approaches. *Sensors*, 15(7), 15684-15716.

- Veerakumar, P., Veeramani, V., Chen, S. M., Madhu, R., & Liu, S. B. (2016). Palladium nanoparticle incorporated porous activated carbon: electrochemical detection of toxic metal ions. *ACS Applied Materials and Interfaces*, 8(2), 1319-1326.
- Wang, L., Chen, S., Xu, F., Chen, S., Jia, J., Tan, H., Hou, H., & Song Y. (2014). Electrochemical sensing and biosensing platform based on biomass-derived macroporous carbon materials. *Analytical Chemistry*, 86, 1414–1421.
- Wang, P., Wang, X., Wang, L., Hou, X., Liu, W., & Chen, C. (2015). Interaction of gold nanoparticles with proteins and cells. *Science and Technology of Advanced Materials*, 16(3), 034610-034625.
- Wang, X., Gu, M., Toh, T. B., Abdullah, N. L. B., & Chow, E. K. H. (2018). Stimuli-responsive nanodiamond-based biosensor for enhanced metastatic tumor site detection. *SLAS TECHNOLOGY: Translating Life Sciences Innovation*, 23(1), 44-56.
- Wang, Z., & Dai, Z. (2015). Carbon nanomaterial-based electrochemical biosensors: an overview. *Nanoscale*, 7(15), 6420-6431.
- Wardani, G., & Sudjarwo, S. A. (2018). In vitro antibacterial activity of chitosan nanoparticles against Mycobacterium tuberculosis. *Pharmacognosy Journal*, 10(1), 162-166.
- Watterson D, Modhiran N., & Young PR. (2016). The many faces of the flavivirus NS1 protein offer a multitude of options for inhibitor design. *Antiviral Research*, 130, 7–18.
- WHO. (2015). Immunization, Vaccines, Biologicals: Japanese Encephalitis. World Health Organisation, Geneva.
- Wikipedia Contributors. (2020, January 10). Silver chloride electrode. In *Wikipedia, The Free Encyclopedia*. Retrieved 07:45, February 20, 2020,

- from https://en.wikipedia.org/w/index.php?title=Silver_chloride_electrode&oldid=935149519. [Assessed on 18 February 2020]
- Wu, S., He, Q., Tan, C., Wang, Y., & Zhang, H. (2013). Graphene-based electrochemical sensors. *Small*, 9(8), 1160-1172.
- Xu, L., Xie, S., Du, J., & He, N. (2017). Porous Magnetic Pseudo-Carbon Paste Electrode Electrochemical Biosensor for DNA Detection. *Journal of Nanoscience and Nanotechnology*, 17(1), 238-243.
- Yadav, B. C., & Kumar, R. (2008). Structure, properties and applications of fullerenes. *International Journal of Nanotechnology and Applications*, 2(1),15-24.
- Yadav, G., Pathak, G., Agrahari, K., Kumar, M., Khan, M. S., Chandel, V. S., & Manohar, R. (2019). Improved dielectric and electro-optical parameters of nematic liquid crystal doped with magnetic nanoparticles. *Chinese Physics B*, 28(3), 034209-034231.
- Yamamoto, T., Moriwaki, Y., & Takahashi, S. (2005). Effect of ethanol on metabolism of purine bases (hypoxanthine, xanthine, and uric acid). *Clinica Chimica Acta*, 356(1-2): 35-57.
- Yamanaka, K., Vestergaard, M. D., & Tamiya, E. (2016). Printable electrochemical biosensors: a focus on screen-printed electrodes and their application. *Sensors*, 16(10), 1761-1777.
- Yan, M., Zang, D., Ge, S., Ge, L., & Yu, J. (2012). A disposable electrochemical immunosensor based on carbon screen-printed electrodes for the detection of prostate specific antigen. *Biosensors and Bioelectronics*, 38(1), 355-361.
- Yáñez-Sedeño, P., Campuzano, S., & Pingarrón, J. (2017). Fullerenes in electrochemical catalytic and affinity biosensing: A review. *Carbon*, 3(3), 21-35.

- Yang, D.K., Kweon, C.H., Kim, B.W., Hwang, I.J., Kang, M.I., So, B.J., & Cho, K.O. (2007). The seroprevalence of Japanese encephalitis virus in goats raised in Korea. *Journal of Veterinary Science*, 8, 197–199.
- Yang, J., Ma, Z., Gao, W., & Wei, M. (2017). Layered Structural Co-Based MOF with Conductive Network Frames as a New Supercapacitor Electrode. *Chemistry – A European Journal*, 23(3), 631-636.
- Yang, K., Feng, L., Hong, H., Cai, W., & Liu, Z. (2013). Preparation and functionalization of graphene nanocomposites for biomedical applications. *Nature Protocols*, 8(12), 2392-2403.
- Yang, X., Fu, T., Kota, P., Tjia, M., Nguyen, C., & Chiao, J. C. (2016). Lactate sensors on flexible substrates. *Biosensors*, 6(3), 48-60.
- Yang, Y., Cui, J., Zheng, M., Hu, C., Tan, S., Xiao, Y., Yang, Q., & Liu, Y. (2012). One-step synthesis of amino-functionalized fluorescent carbon nanoparticles by hydrothermal carbonization of chitosan. *Chemical Communications*, 48(3), 380-382.
- Yazid, S. N. A. M., Chin, S. F., Pang, S. C., & Ng, S. M. (2013). Detection of Sn (II) ions via quenching of the fluorescence of carbon nanodots. *Microchimica Acta*, 180(1-2), 137-143.
- Yeh, B. Y., Jian, B. S., Wang, G. J., & Tseng, W. J. (2017). CuO/V₂O₅ hybrid nanowires for highly sensitive and selective H₂S gas sensor. *RSC Advances*, 7(78), 49605-49612.
- Yeh, J. Y., Lee, J. H., Seo, H. J., Park, J. Y., Moon, J. S., Cho, I. S., & Choi, I. S. (2010). Fast duplex one-step reverse transcriptase PCR for rapid differential detection of West Nile and Japanese encephalitis viruses. *Journal of Clinical Microbiology*, 48(11), 4010-4014.

- Yoo, E. H., & Lee, S. Y. (2010). Glucose biosensors: an overview of use in clinical practice. *Sensors*, 10(5), 4558-4576.
- Yun, S.I., & Lee, Y.M. (2014). Japanese encephalitis; the virus and vaccines. *Human Vaccines & Immunotherapeutics*, 10(2), 263-279.
- Zanin, M.P., Webster, D.E., Martin, J.L., & Wessenlingh, S.L. (2003). Japanese encephalitis vaccines: moving away from mouse brain. *Expert Review of Vaccines*, 2, 407–416.
- Zanini, V. P., de Mishima, B. L., & Solís, V. (2011). An amperometric biosensor based on lactate oxidase immobilized in laponite–chitosan hydrogel on a glassy carbon electrode. Application to the analysis of l-lactate in food samples. *Sensors and Actuators B: Chemical*, 155(1), 75-80.
- Zeng, Q., Shao, D., He, X., Ren, Z., Ji, W., Shan, C., & Li, Q. (2016). Carbon dots as a trackable drug delivery carrier for localized cancer therapy in vivo. *Journal of Materials Chemistry B*, 4(30), 5119-5126.
- Zhang, J. X., & Hoshino, K. (2018). *Molecular Sensors and Nanodevices: Principles, Designs and Applications in Biomedical Engineering*, 2nd ed., UK: Elsevier Inc.
- Zhang, L., Li, S., Xin, J., Ma, H., Pang, H., Tan, L., & Wang, X. (2019). A non-enzymatic voltammetric xanthine sensor based on the use of platinum nanoparticles loaded with a metal-organic framework of type MIL-101 (Cr). Application to simultaneous detection of dopamine, uric acid, xanthine and hypoxanthine. *Microchimica Acta*, 186(1), 9-29.
- Zhao, Y., Liu, M., Deng, X., Miao, L., Tripathi, P. K., Ma, X., ... & Gan, L. (2015). Nitrogen-functionalized microporous carbon nanoparticles for high performance supercapacitor electrode. *Electrochimica Acta*, 153, 448-455.
- Zhao, G. L., Gao, F., & Wang, Z. (2018). U.S. Patent No. 9,944,528. Washington, DC: U.S. Patent and Trademark Office.

- Zheng, L., Cai, G., Wang, S., Liao, M., Li, Y., & Lin, J. (2019). A microfluidic colorimetric biosensor for rapid detection of *Escherichia coli* O157: H7 using gold nanoparticle aggregation and smart phone imaging. *Biosensors and Bioelectronics*, 124, 143-149.
- Zheng, X., Li, L., Cui, K., Zhang, Y., Zhang, L., Ge, S., & Yu, J. (2018). Ultrasensitive enzyme-free biosensor by coupling cyclodextrin functionalized au nanoparticles and high-performance au-paper electrode. *ACS Applied Materials and Interfaces*, 10(4), 3333-3340.
- Zhou, D. D. (2008). Microelectrodes for in-vivo determination of pH. Electrochemical sensors, biosensors and their biomedical applications. *Academic Press*, USA, 261-305.
- Zhu, J., Gan, H., Wu, J., & Ju, H. (2018). Molecular machine powered surface programmatic chain reaction for highly sensitive electrochemical detection of protein. *Analytical Chemistry*, 90, 5503–5508.
- Zou, X., Wu, J., Gu, J., Shen, L., & Mao, L. (2019). Application of Aptamers in Virus Detection and Antiviral Therapy. *Frontiers in Microbiology*, 10, 1462-1477.

APPENDICES

Journal Publications

1. **Lai, H. C.**, Chin, S. F., Pang, S. C., Sum, H., Sia, M., & Perera, D. (2017). Carbon nanoparticles based electrochemical biosensor strip for detection of Japanese Encephalitis Virus. *Journal of Nanomaterials*, 2017, 1-7.
2. Chin, S. F., Lim, L. S., **Lai, H. C.**, Pang, S. C., Sum, M. S. H., & Perera, D. (2019). Electrochemical Determination of Japanese Encephalitis Virus Antigen Using Silver Nanoparticles Modified Screen-Printed Carbon Electrode. *Nano Biomedical Engineering*, 11(4), 333-339.

Patent

1. Chin, S.F. & **Lai, H.C.** (2015). Novel and Green Fabrication Process. Portable Nano-Biosensor for Rapid & Sensitive Detection of Japanese Encephalitis Virus Antigen. Malaysia. Patent Pending (PI 2015703).

Participation in EXPO

1. Chin, S. F., **Lai, H. C.**, Pang, S. C., Sum, H., Sia, M., & Perera, D. (2016). **Gold Medal**, Innovation Technology Expo 2016 (InTEX 16), *Portable Nano-Biosensor for Rapid & Sensitive Detection of Japanese Encephalitis Virus Antigen*.
2. Chin, S. F., **Lai, H. C.**, Pang, S. C., Sum, H., Sia, M., & Perera, D. (2016). **Silver Medal**, Seoul International Invention Fair 2016 (SIIF 2016). *Portable Nano-Biosensor for Rapid & Sensitive Detection of Japanese Encephalitis Virus Antigen*.