UNIVERSITÉ DU QUÉBEC À MONTRÉAL

DEVELOPMENT OF ELECTROCHEMICAL IMMUNOSENSORS FOR THE FEMTOMOLAR DETECTION OF DIAGNOSTIC BIOMARKERS

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EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
PDITC	1,4-phenylene diisothiocyanate
TMB	3,3',5,5'-Tetramethyl-benzidine
GAA	Acid a-glucosidase
AC	Alternating current
BSA	Bovine serum albumin
R_{ct}	Charge transfer resistance
CT scans	Computed Tomography Scan
CPE	Constant phase element
CV	Cyclic Voltammetry
DHF	Dengue hemorrhagic fever
DSS	Dengue shock syndrome
DENV	Dengue Virus
DMF	Dimethylformamide

EIS	Electrochemical impedance spectroscopy
ERT	Enzyme replacement therapy
ELISA	Enzyme-linked immunosorbent assay
FRA	Frequency response analyzer
GALC	Galactocerebrosidase
GC	Glassy carbon
GCL	Globoid cell leukodystrophy
AuNPs	Gold nanoparticles
Au-S	Gold–Sulphur
HRP	Horseradish Peroxidase
IKZF1	Ikaros family zinc finger 1
IKZF3	Ikaros family zinc finger 3
IMiDs	Immunomodulatory imide drug
ITO	Indium Tin Oxide
KD	Krabbe disease

LOC	Lab-on-a-chip
LOD	Limit of detection
LC-MS/MS	Liquid chromatography–mass spectrometry
LSDs	Lysosomal storage disorders
MRI	Magnetic resonance imaging
MM	Multiple myeloma
NS1	Non-structural protein 1
ΔE	Peak-to-peak separation
PBS	Phosphate buffer saline
POCT	Point-of-care testing
RSD %	Relative standard deviation
RT-PCR	Reverse transcriptase-polymerase chain reaction
SEM	Scanning electron microscopy
SPCE	Screen-printed carbon electrode
SPEs	Screen-printed electrodes

SPGE	Screen-printed graphite electrode
SAM	Self-assembled monolayer
R_s	Solution resistance
SWV	Square wave voltammetry
Z_W	Warburg Impedance
WB	Western Blot
GBA	β-Glucocerebrosidase

RESUMÉ

Le diagnostic précoce des maladies est crucial afin de prévenir le développement des symptômes invalidants en permettant une intervention et un traitement plus à temps. Cette approche est porteuse d'espoir puisqu'une détection plus rapide, idéalement par l'identification de signes de propagation, pourrait permettre de prévenir l'apparition de la maladie, en ayant recours à des traitements qui empêcheraient son développement. Cela conduira à de meilleurs résultats pour le patient. Lorsqu'une maladie est détectée à un stade précoce, les chances de succès du traitement et de guérison sont généralement beaucoup plus élevées que si la maladie est détectée à un stade ultérieur, cela peut également aider à réduire le coût global du traitement. De plus, les patients qui reçoivent un diagnostic précoce peuvent prendre des décisions plus éclairées concernant leur traitement et ont également de meilleures chances de reprendre leurs activités quotidiennes normales. La détection et la surveillance précoces des médicaments anticancéreux sont également essentielles, car elles permettent d'optimiser le traitement et garantissent la sécurité des patients. Dans ce sens, il est important de développer de nouvelles méthodes de diagnostic afin d'améliorer les chances de succès du traitement et de la guérison, à réduire le coût global des soins de santé et à améliorer la qualité de vie des patients. Cela peut conduire à un système de santé plus efficient et efficace qui peut aider à sauver des vies et à améliorer la santé publique.

La haute sensibilité, la spécificité et la rentabilité des biocapteurs, ainsi que leur potentiel d'intégration avec d'autres technologies, en font un intérêt de recherche majeur dans le domaine du diagnostic précoce des maladies. Dans cette thèse, nous nous sommes concentrés sur le développement de biocapteurs électrochimiques pour la détection, la surveillance et le diagnostic précoce des maladies. Des immunocapteurs électrochimiques ultra-sensibles et sélectifs ont été développés et testés avec succès dans le sérum humain.

Premièrement, pour surveiller les patients atteints de myélome multiple (MM) traités avec l'agent chimiothérapeutique lénalidomide et pour prévenir la cytotoxicité issue du médicament et de minimiser ses effets secondaires, un nouvel immunocapteur électrochimique sans marquage a été

développé pour la détection et la quantification rapides des protéines à doigt de zinc 1 et 3 de la famille Ikaros (IKZF1 et IKZF3). Une expression élevée de ces protéines dans le MM entraîne une moindre sensibilité au traitement par le lénalidomide et d'éventuels effets cytotoxiques. Par conséquent, la détection des protéines IKZF1 et IKZF3 est cruciale dans le traitement du MM.

Deuxièmement, un immunocapteur électrochimique pour la quantification multiplexée pour une détection simultanée de acid α -glucosidase (GAA), β-Glucocerebrosidase (GBA) et la galactocerebrosidase (GALC) a été développé avec succès. Cet immunocapteur affiche une sensibilité à l'échelle femtomolaire jumelée à une bonne sélectivité et un bon temps de récupération pour revenir à la valeur de référence après la suppression progressive de la variable mesurée lorsqu'il est enrichi avec du sérum humain. Ses caractéristiques confirent la possibilité d'une utilisation potentielle du présent capteur dans les tests au point de service pour une identification précoce des troubles du stockage lysosomal (LSD).

Enfin, nous présentons le développement d'un immunocapteur électrochimique sans marqueur pour la quantification simultanée et multiplexée de quatre sérotypes différents de l'antigène NS1 du virus de la dengue (DENV-1, DENV-2, DENV-3 et DENV-4) au stade précoce. Les immunocapteurs électrochimiques nouvellement fabriqués offrent des alternatives prometteuses aux méthodes de diagnostic actuellement utilisées et sont très prometteurs en tant que dispositifs de test potentiels au point de service pour le diagnostic à un stade précoce.

ABSTRACT

Early diagnosis of diseases is crucial as it allows for earlier intervention and treatment, which can lead to better outcomes for the patient. When a disease is detected at an early stage, the chances of successful treatment and recovery are typically much higher than if the disease is detected at a later stage, it can also help to reduce the overall cost of treatment. Furthermore, patients who are diagnosed early can make more informed decisions about their treatment, and also have a better chance of returning to their normal daily activities. Early detection and monitoring of cancer drugs is also essential because it allows for the optimization of treatment and ensures patient safety. Developing new methods for early diagnosis of diseases that are more sensitive and cost-effective is important as it can help improve the chances of successful treatment and recovery, reduce the overall cost of healthcare, and improve the quality of life for patients. This can lead to a more efficient and effective healthcare system that can help save lives and improve public health.

The high sensitivity, specificity and cost-effectiveness of biosensors, as well as their potential for integration with other technologies, make them a major research interest in the field of early diagnosis, as they can offer a powerful tool to detect diseases at an early stage and improve the chances of successful treatment and recovery. In this dissertation, we focused on developing electrochemical biosensors for the detection, monitoring, and early diagnosis of diseases. Ultra-sensitive and selective electrochemical immunosensors were successfully developed and tested in human serum.

First, for monitoring multiple myeloma patients treated with chemotherapeutic agent Lenalidomide, to prevent the drug's cytotoxicity and minimize its side effects, a novel label-free electrochemical immunosensor was developed for the rapid detection and quantification of Ikaros family zinc finger proteins 1 and 3 (IKZF1 and IKZF3). High expression of these proteins in MM results in less sensitivity to lenalidomide treatment and possible cytotoxic effects. Therefore, detecting IKZF1 and IKZF3 proteins is crucial in the treatment of MM.

Second, an electrochemical immunosensor for the multiplexed quantification and simultaneous detection of GAA, GBA and GALC was successfully developed. This multiplexed immunosensor was able to successfully detect GAA, GBA and GALC at the femtomolar level and demonstrated good selectivity, sensitivity and good recovery when spiked in human serum, confirming its potential use in point-of-care testing for the early identification of lysosomal storage disorders (LSDs).

Finally, we present the development of a label-free electrochemical immunosensor for the simultaneous and multiplexed quantification of four different serotypes of Dengue virus NS1 antigen (DENV-1, DENV-2, DENV-3 and DENV-4) at the early stage. The newly fabricated electrochemical immunosensors offer promising alternatives to the currently used diagnostic methods and hold great promise as a potential point-of-care testing devices for early-stage diagnosis.

CHAPTER 1

INTRODUCTION

1.1 Medical Diagnostics

Medical diagnostic techniques have come a long way over the years. To make a diagnosis in the past, doctors used a combination of physical examination, patient history, and their own experience. In order to arrive at a diagnosis, the doctor would frequently utilize a process of elimination in which they would weigh various options and attempt to rule out plausible reasons.

This method's primary drawback was that it was frequently challenging to make a definitive diagnosis of some conditions. For instance, it could be difficult to distinguish between several illness kinds or to pinpoint the origin of some symptoms. As a result, treatment decisions were frequently dependent on educated assumptions, and results could vary.

Medical diagnostic methods have advanced significantly in sophistication and dependability in recent years. Our capacity to correctly identify a variety of disorders has significantly improved thanks to the development of new technologies such as imaging techniques (e.g., CT scans, MRI), laboratory testing (e.g., blood tests, genetic testing), and enhanced diagnostic devices (e.g., electrocardiograms, endoscopes). These methods give medical professionals the ability to see into the body, identify specific abnormalities or indicators, and gain a more thorough grasp of a patient's health. Patients' outcomes have greatly improved as a result of the treatment becoming more targeted and effective (Nemčeková et Labuda, 2021).

1.2 Point-of-care testing (POCT)

Point-of-care testing (POCT) refers to the use of diagnostic tests that are performed at or near the site of patient care, rather than in a central laboratory. The quick results that can be utilized to guide patient treatment decisions are provided by POCT devices, which are portable and

simple to use. A variety of locations, including hospitals, clinics, doctor's offices, and at-home testing, use POCT equipment (Figure 1.1) (Srinivasan et Tung, 2015).

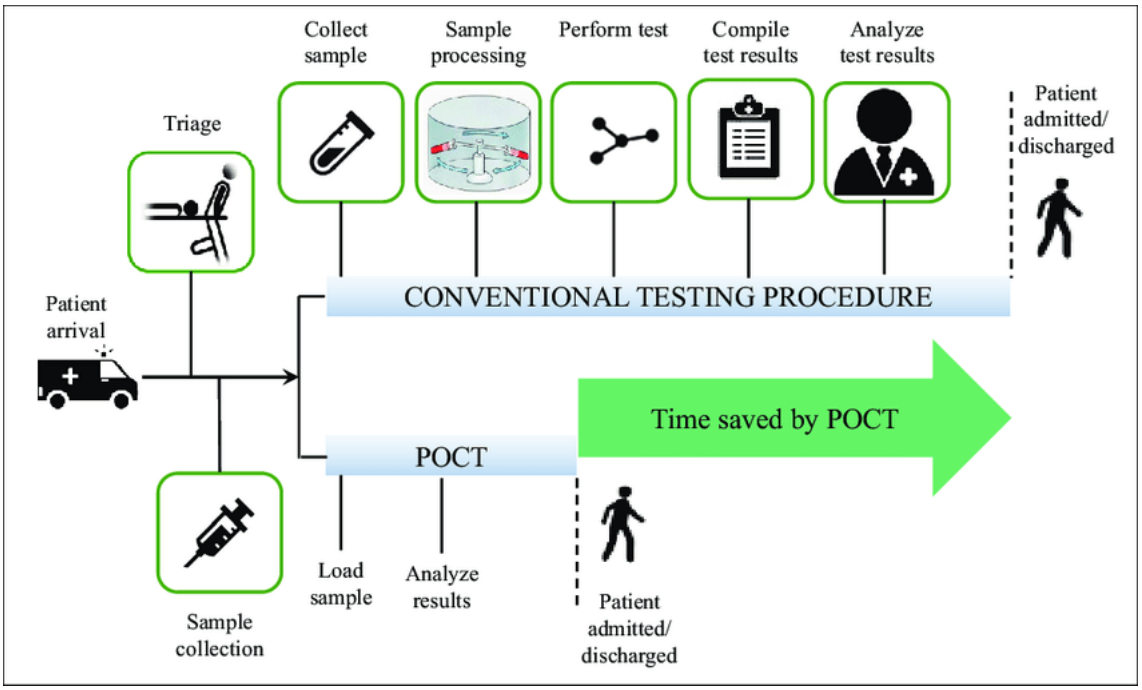

Figure 1.1 Rapid turnaround of results with point-of-care testing POCT compared to the conventional testing procedure. Reproduced from reference (Srinivasan et Tung, 2015).

There are many different kinds of POCT devices, including those that employ immunoassays, molecular approaches, and other analytical techniques. POCT devices can be used to test for a variety of analytes, such as infections, hormones, medications, and other biomarkers.

One of the main advantages of POCT is that it allows for faster diagnosis and treatment of patients, as the results are available much sooner than if the samples were sent to a central laboratory for testing.This may be particularly significant in critical care situations, where prompt identification and treatment may mean the difference between life and death. The fact that POCT does not require patients to travel to a lab for sample collection and testing means that it may be more convenient for them.

The use of POCT presents a number of difficulties, but one of these is the requirement for skilled individuals to use the equipment and analyze the data. Although technological advancements have enhanced the performance of many POCT devices, they could not be as accurate or exact as central laboratory testing. Last but not least, there are concerns regarding the correct handling and storage of POCT samples as well as the requirement for quality control and equipment maintenance (Choi, 2020).

1.3 Biosensors

Biosensors are analytical devices that use biological components, known as bioreceptors, to selectively detect and quantify a specific analyte in a sample. The bioreceptor can be a microorganism, enzyme, antibody, or other biomolecule that is capable of recognizing and binding to the target analyte (Figure 1.2) (Farzin *et al.*, 2018). Applications for biosensors are numerous and include environmental monitoring, food safety testing, and medical diagnostics. They have been employed as diagnostic tools for a long time, and over that period, they have undergone a number of important modifications in development.

One of the earliest examples of a biosensor was the glucose biosensor, which was created in the 1950s employing enzymes as the biological component. A glass electrode coated with an enzyme that could change glucose into gluconic acid was the basic component of these early glucose biosensors. The electrode recognized the pH shift brought on by this reaction, and the resulting signal was used to calculate the amount of glucose present in the sample.

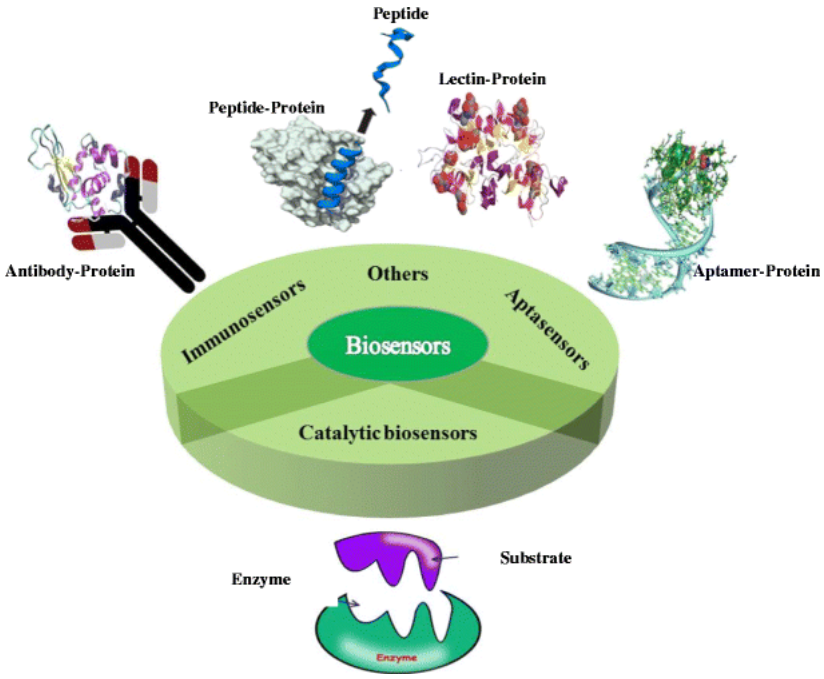

Figure 1.2 The main classes of bioreceptors used in the biosensing applications. Reproduced from reference (Farzin et al., 2018)

The use of enzymes as the biological component started to receive increasing attention in the 1970s and 1980s as biosensor research progressed. This was partly a result of the increased knowledge of the function of enzymes in biochemical processes and the expansion of enzymes with particular functions. Enzymes can detect a large range of compounds with great specificity and sensitivity, making them ideal for use in biosensors.

More recently, the development of biosensors has focused on the use of other biological components, such as antibodies, nucleic acids, and cells, to detect and measure specific substances. These elements have made it possible to create more sophisticated biosensors that can detect a wider variety of compounds and give more in-depth details about the sample being studied.

Additionally, technological advancements have made it possible to create portable and handheld biosensors that may be utilized in a variety of locations, including clinics, hospitals, and even homes. These portable biosensors are useful instruments for diagnostic purposes since they can deliver prompt and precise data. In general, improvements in our comprehension of biological processes and the development of new technologies that have made it feasible to produce more sensitive and specific biosensors have been the driving forces behind the advancement of biosensors as diagnostic tools (Cesewski et Johnson, 2020; Naresh et Lee, 2021).

Biosensors have several advantages over other diagnostic tools that make them attractive for use in a variety of applications (Kaya *et al.*, 2021). Sensitivity is one of the key advantages offered by biosensors; this ability to detect minimal levels of a particular analyte makes biosensors particularly sensitive. Because of this, they are especially helpful for detecting traces of analytes in complicated samples like blood, serum, or urine. The ability to discriminate between different compounds even when they are present at equal amounts is another benefit of biosensors. They are able to detect specific analytes with high specificity. They can therefore be helpful in identifying particular biomarkers or signs of a given condition or disease. Biosensors provide rapid results, often within minutes or hours, depending on the specific application. This makes them useful for situations where quick results are needed, such as in emergency care or public health surveillance. Depending on the individual application, biosensors frequently produce data in minutes or hours. They are therefore helpful in circumstances requiring speedy outcomes, such as emergency care or public health surveillance. The fact that many biosensors are made to be used by non-technical persons and are frequently portable and simple to use makes them advantageous as well. This makes them useful for usage in a number of contexts, such as hospitals, clinics, and even homes. A cost-effective diagnostic tool, biosensors can be produced and used for comparatively low costs. This is crucial in environments with few resources because using conventional diagnostic techniques could be expensive. The majority of biosensors are also non-invasive, meaning that they don't need intrusive procedures like blood draws to collect samples for analysis. They become less dangerous and more practical for patients as a result, especially for repeated testing. Overall, biosensors offer a number of advantages over other diagnostic tools, making them valuable for a wide range of applications (Haleem *et al.*, 2021).

1.3.1 Electrochemical immunosensors

An electrochemical immunosensor is a type of biosensor that uses an electrochemical transducer to detect the presence of a specific antigen or antibody in a sample. A biomolecule, such as an antibody or antigen, is often added to the transducer to generate a sensing element. The transducer is typically a conductive substance, such as gold or carbon. A change in the transducer's electrical properties occurs when the sample is added because the immobilized antigen or antibody in the sample binds to it. An electrical current that passes through the transducer can be used to identify and measure this change (Mollarasouli *et al.*, 2019).

Compared to other kinds of biosensors, electrochemical immunosensors have a number of advantages. They have a very high sensitivity and can detect the target analyte at very low quantities. They can be easily integrated into portable or handheld equipment for on-site testing and are also fairly quick and simple to use. Electrochemical immunosensors are also suited for a variety of applications due to their inexpensive cost and ability to detect a wide spectrum of analytes.

There are several different types of electrochemical immunosensors, including amperometric, potentiometric, and conductometric immunosensors. Amperometric immunosensors use an electrical current to measure the change in the current flow through the transducer as the analyte binds to the bioreceptor. Potentiometric immunosensors use a voltage difference to measure the change in the electrical potential of the transducer as the analyte binds to the bioreceptor. Conductometric immunosensors use the change in the electrical conductivity of the transducer as the analyte binds to the bioreceptor. An electrochemical immunosensor is a valuable tool for detecting and quantifying specific analytes in a sample and have a wide range of applications in various fields (Kim et Park, 2021).

1.3.2 Electrode Types

Various electrode types can be utilized in biosensors, depending on the particular application and analysis technique. Electrode materials, electrode geometries, and electrode surfaces are some typical categories to take into account when deciding what electrodes to choose when constructing biosensors. Electrodes made of metals (such as gold, platinum, and carbon),

semiconductors (such as silicon and tin oxide), and insulators (such as alumina) can all be used as electrodes in biosensors. The kind of analyte to be detected, the required sensitivity and specificity, and the operating circumstances will all have an impact on the electrode material selection. Planar, cylindrical, and spherical geometries are some of the possible designs for electrodes. The sensitivity, specificity, and ease of production and integration into a device of the biosensor can all be influenced by the geometry of the electrode. To enhance the performance of the biosensor, the surface of the electrode can also be modified with different materials. To increase the electrical conductivity and stability of the sensor, for instance, a layer of gold nanoparticles (AuNPs) can be applied to the electrode surface. In order to generate a sensing component, the electrode surface can also be functionalized with biomolecules like enzymes or antibodies.

In general, the kind of electrode that is utilized in a biosensor will rely on the particular specifications and analytical approach of the sensor. The performance, sensitivity, and specificity of the biosensor can be enhanced for a given application by using several types of electrodes.

1.3.3 Nanomaterials in Biosensors

Nanomaterials can considerably increase the sensitivity of biosensors by offering a platform for highly sensitive and selective detection. To obtain high sensitivity and selectivity while preserving stability and durability is one of the primary problems in the design of biosensors. Nanomaterials can aid in overcoming these difficulties by offering a platform for extremely sensitive and selective detection. The signal produced by the biosensor, for instance, can be amplified using nanomaterials like nanoparticles and nanostructures, enabling the detection of biomolecules at incredibly low concentrations (Figure 1.3) (Sheikhzadeh *et al.*, 2021). Additionally, they can be utilized to selectively capture specific target biomolecules, enhancing the biosensor's selectivity.

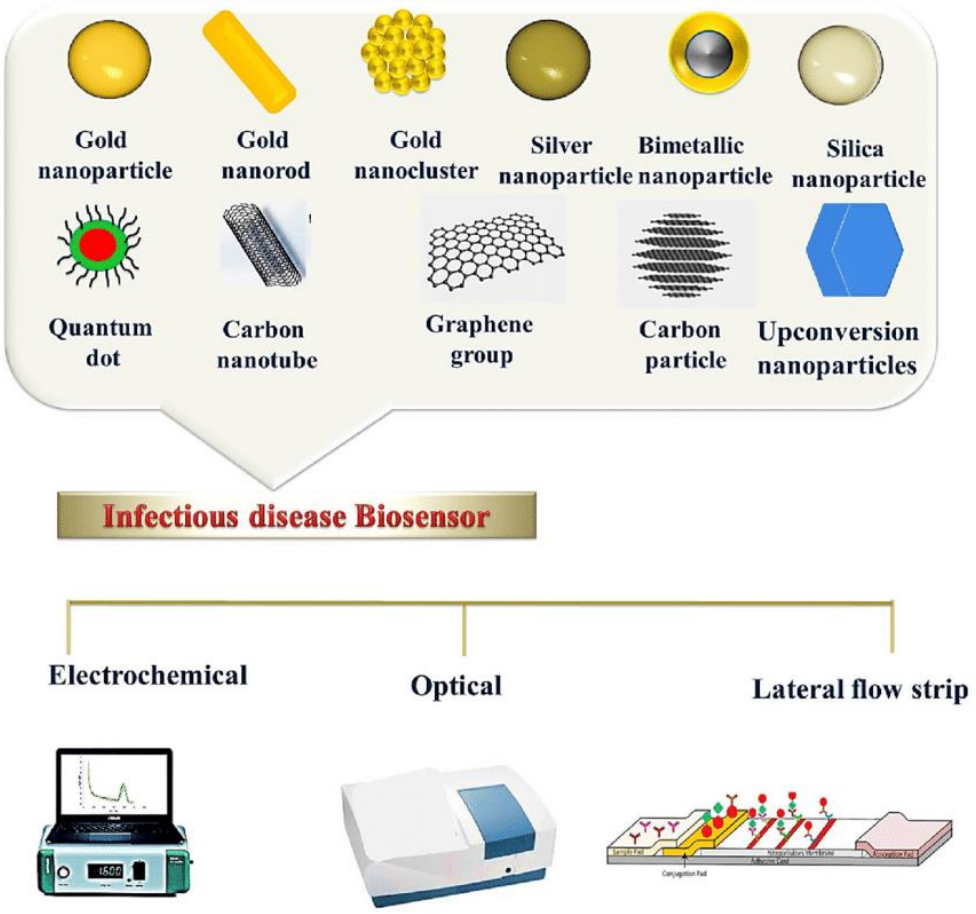

Figure 1.3 The use of different nanomaterials in biosensors. Reproduced from reference (Sheikhzadeh et al., 2021)

Nanomaterials can also be utilized to stabilize the biosensor and increase its reliability. They can be employed, for instance, to enhance the stability of the sensing mechanism or to shield the biological element of the biosensor from degradation. Nanomaterials have the potential to dramatically increase the sensitivity, selectivity, stability, and reliability of biosensors, making them more practical and efficient in a variety of applications.

1.3.4 Electrodeposition of Gold Nanoparticles (AuNPs)

The deposition of gold nanoparticles (AuNPs) is an important step in the development of biosensors, as it can improve the performance and sensitivity of the sensor. AuNPs are often used as a transducer material in biosensors due to their unique optical and electrical properties. AuNPs are highly conductive and can be easily functionalized with biomolecules, such as enzymes or antibodies, to create a sensing element. The functionalized AuNPs can be immobilized on a solid support, such as a microplate or electrode, to create a biosensor.

The deposition of AuNPs on the solid support is typically achieved through a process known as electrodeposition, in which the AuNPs are reduced to a metallic form in the presence of a reducing agent. The AuNPs can be functionalized with a variety of biomolecules, depending on the specific application of the biosensor. The deposition of AuNPs is an important step in the development of biosensors, as it allows for the creation of a highly sensitive and specific sensing element. In addition, the use of AuNPs allows for the integration of the biosensor into portable or handheld devices, making it suitable for on-site testing. Generally, the deposition of AuNPs is a key factor in the development of effective and sensitive biosensors.

1.3.5 Electrodeposition of Gold Nanoparticles (AuNPs) via chronoamperometry

Chronoamperometry is a technique used to measure the current flowing through an electrode as a function of time. It is commonly used in electrochemistry to study the kinetics of electrochemical reactions and to determine the rate of electron transfer between a redox species and an electrode.

In a chronoamperometric measurement, an electrochemical cell is set up with a working electrode, a reference electrode, and a counter electrode. The working electrode is the electrode at which the electrochemical reaction of interest occurs, while the reference and counter electrodes are used to complete the circuit and provide a stable potential (Figure 1.4) (Damiati et Schuster, 2020).

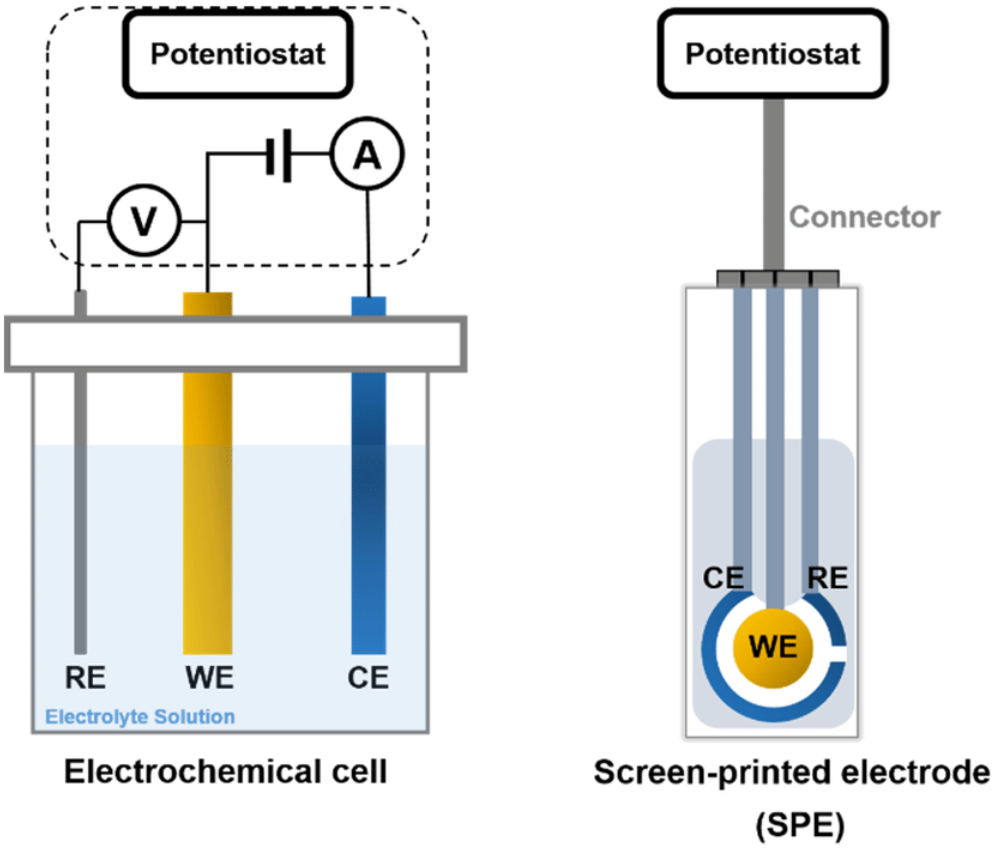

Figure 1.4 The two types of electrochemical biosensors with three electrodes: reference (RE), working (WE), and counter (CE) connected to a potentiostat. Reproduced from reference (Damiati et Schuster, 2020)

The potential of the working electrode is typically controlled by a potentiostat, which is a device that allows the potential of the working electrode to be set and maintained at a specific value relative to the reference electrode.

A constant potential is applied to the working electrode to make a chronoamperometric measurement. The resulting current is then measured at regular intervals over a predetermined amount of time. The current is typically measured using a high-impedance ammeter, which is able to measure small currents without significantly affecting the potential of the working electrode.

The chronoamperogram that results from this current-time curve tells us more about the kinetics of the electrochemical process taking place at the working electrode. The chronoamperometric response, which is a measurement of the rate of the electrochemical reaction, is the slope of the curve at a specific instant in time. Chronoamperometry is a practical method with several uses, such as corrosion research, electrocatalyst characterisation, and kinetic parameter measurement in electrochemical systems. It is frequently employed in the electrodeposition method of producing gold nanoparticles (AuNPs).

Electrodeposition of gold nanoparticles (AuNPs) via chronoamperometry is a technique used to synthesize AuNPs using an electrochemical cell and a potentiostat. It involves the reduction of gold ions in solution to form AuNPs at a working electrode, which is typically made of gold or another conductive material. Preparation of the electrochemical cell, potentiostatic control, chronoamperometric measurements, and the electrodeposition of AuNPs are usually the steps involved in the electrodeposition of AuNPs through chronoamperometry.

The electrochemical cell is first set up by placing the working electrode in the cell and filling it with the electrolyte solution containing gold ions. The reference and counter electrodes are also placed in the cell. The potentiostat is used to control the potential of the working electrode relative to the reference electrode. This is typically done by applying a constant potential to the working electrode and measuring the resulting current. Moreover, chronoamperometric measurement is used to measure the current at the working electrode as a function of time. This involves applying a constant potential to the working electrode and measuring the current at

regular intervals over a specific period of time. During the chronoamperometric measurement, gold ions in the electrolyte solution are reduced at the surface of the working electrode, forming AuNPs. The size and shape of the AuNPs can be controlled by adjusting the potential applied to the working electrode and the duration of the measurement.

Generally, the electrodeposition of AuNPs via chronoamperometry is a simple and effective way to synthesize AuNPs with precise control over the size and shape of the particles. It is widely used in the production of AuNPs for a variety of applications, including biosensors, catalysts, and drug delivery.

1.4 Electrochemical techniques

1.4.1 Cyclic voltammetry (CV)

Cyclic voltammetry (CV) is an electrochemical technique that is used to study the reduction and oxidation reactions that occur at an electrode. It involves applying a series of voltage pulses to an electrochemical cell and measuring the resulting current. In CV, the electrode's potential is linearly swept from a high initial potential to a lower final potential, and back again. The potential is typically altered at a constant rate called the sweep rate. A plot of the current versus potential is obtained after the current flowing through the cell is measured during the sweep. This plot is known as a voltammogram.

CV has several uses in the realm of biosensors and is an important tool for researching electrochemical reactions. When it comes to biosensors, CV can be used to examine the electrochemical characteristics of the biological element, such as an enzyme or a cell layer, that makes up the sensor. Researchers can learn more about the kinetics and mechanism of the biological events occurring inside the sensor by measuring the current flowing through the sensor at various potentials (Venton et Cao, 2020).

CV is often used in combination with other electrochemical techniques, such as chronoamperometry and electrochemical impedance spectroscopy, to study the behavior of biosensors in more detail. It is a sensitive and reliable method for studying the electrochemical properties of biosensors, and it is widely used in the development and optimization of these devices.

1.4.2 Square wave voltammetry (SWV)

Square wave voltammetry (SWV) is a type of electrochemical technique that is used to analyze the electrochemical properties of a substance. It is based on voltammetry's fundamental ideas, which measures an electrochemical system's current-voltage (I-V) response as a function of applied potential. In SWV, the electrochemical system is subjected to an alternating potential waveform, and the resulting current is measured. The waveform is made up of a number of potential steps that are each held for a set amount of time. The magnitude and duration of the steps can be varied to enhance the measurement's sensitivity and resolution. The potential steps can alternately be positive and negative.

One of the main advantages of SWV is its high sensitivity and selectivity, as it allows for the detection of trace amounts of analytes. It is also a rapid and reliable method for analyzing electroactive species.

Numerous electrochemical systems, such as redox reactions, corrosion processes, and bioelectrochemical reactions, can be studied using SWV. As it enables the quick and accurate detection of analytes in a range of samples, including biological fluids and environmental samples, it is a frequently employed technology in the field of biosensors (Mirceski *et al.*, 2013).

Overall, SWV is a valuable instrument for examining the electrochemical characteristics of substances and is frequently utilized in the creation of biosensors and other analytical tools.

1.4.3 Electrochemical impedance spectroscopy (EIS)

Electrochemical impedance spectroscopy (EIS) is a measurement technique that is used to study the impedance of a system at different frequencies. Impedance is the opposition that a system offers to the flow of an alternating current (AC). It is the combination of resistance and reactance, which are both measures of how difficult it is for the current to flow through the system. EIS is a non-destructive approach for examining a system's electrical characteristics over a broad frequency range.

Electrochemical systems like batteries, fuel cells, and biosensors are frequently studied using EIS. When it comes to biosensors, EIS can be used to examine the electrical properties of the biological element, such as an enzyme or a cell, that makes up the sensor. Researchers can learn more about the kinetics and mechanism of the biological reactions occurring inside the sensor by measuring the impedance of the biological component at various frequencies.

The electrochemical cell, which comprises of an electrode, a reference electrode, and a counter electrode, is generally used to perform EIS measurements. The cell is given an AC voltage, and the resulting current is then calculated. The magnitude and phase of the current and voltage are then used to compute the impedance of the cell. EIS measurements are usually performed at frequencies ranging from very low (e.g., 10^{-2} Hz) to very high (e.g., 10^{6} Hz). By measuring the impedance at different frequencies, it is possible to obtain information about the different time constants of the system, which can provide insight into the underlying mechanisms of the system (Chang et Park, 2010).

EIS can be used to assess the biological sensor's resistance, such as that of an enzyme or cell. The impedance of the system can be determined by applying an AC voltage to the biosensor and measuring the resulting current. The resistance of the biosensor can then be obtained by taking the real part of the impedance (Instruments, 2007).

EIS can be used for a variety of applications for determining a biosensor's resistance. For instance, it can be used to optimize the biosensor's design in order to increase sensitivity or selectivity. Additionally, it can be utilized to research the stability and longevity of the sensor as well as the kinetics of biological reactions within the sensor (Lisdat et Schäfer, 2008).

EIS is a powerful tool for researching electrochemical systems and has a wide range of uses in the biosensor industry. It can be used to optimize the design of biosensors, analyze the kinetics of biological processes within the sensor, and evaluate the sensor's durability and stability.

1.5 Development of Immunosensors

1.5.1 Labeled vs. Label-Free Immunosensors

Label-based immunosensors detect the desired analyte using a labeled molecule, such as a fluorescent dye or a radioactive isotope. The labeled molecule is often attached to the antigen or antibody employed in the assay, and when it binds to the analyte, a signal is produced. The signal produced by the labeled molecule indicates the presence and/or the concentration of the analyte. In a label-based electrochemical immunosensor, the target analyte is detected by a labeled molecule (such as a redox enzyme or a nanoparticle). The signal from this labeled molecule, which is connected to the antigen or antibody, is detected when it interacts to the analyte. Typically, the signal produced by the labeled molecule is an electrochemical signal that may be detected using voltammetric or amperometric methods. The signal is utilized to provide information regarding the analyte's concentration.

On the other hand, label-free immunosensors do not use labeled molecules, but instead rely on changes in a physical property of the sensor to detect the analyte of interest. Label-free electrochemical immunosensors rely on alterations in the sensor's electrochemical characteristics (such as the current or potential) brought on by the binding of the analyte to the antigen or antibody. Techniques like voltammetry, amperometry, or electrochemical impedance spectroscopy (EIS) can be used to detect these changes (Figure 1.5).

Both types of sensors have their own advantages and disadvantages. The label-based immunosensor is easy to design and has high sensitivity, but the labeling process may affect the activity of antigen or antibody, and the labeled molecule may cause some interference. Label-free immunosensors are free from interference and the activity of antigen or antibody is maintained.

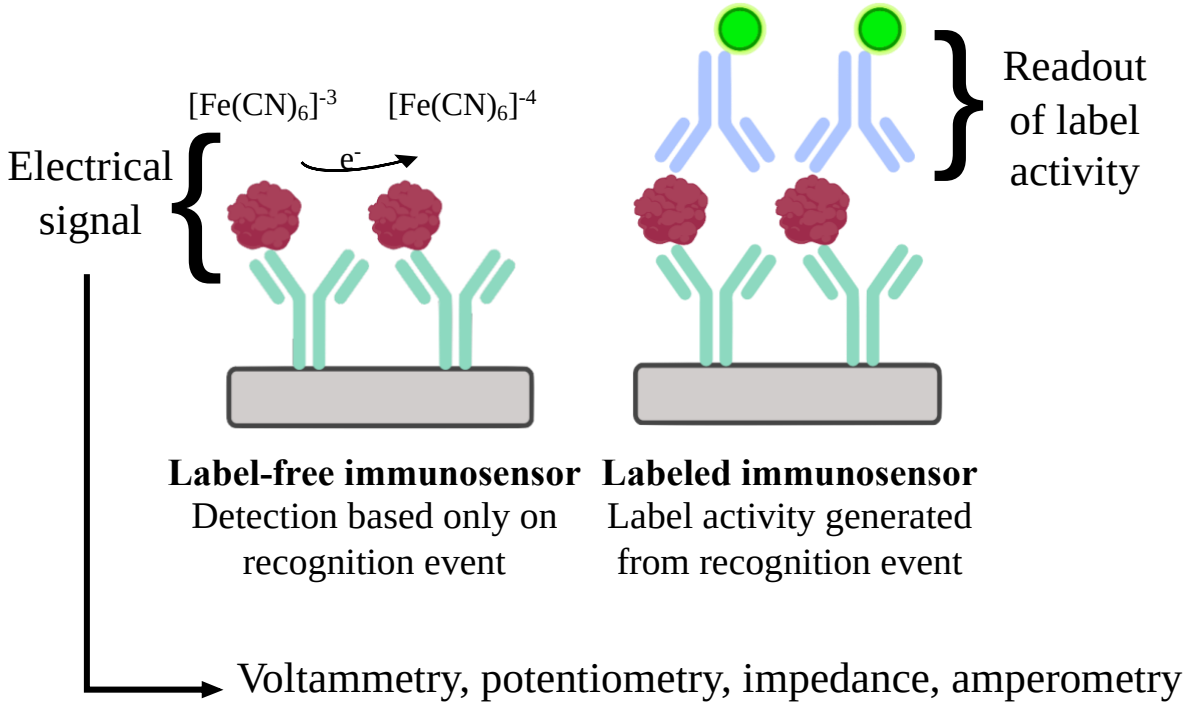

Figure 1.5 Labeled vs. label-free immunosensors.

1.5.2 Sensor Surface Activation and Crosslinking of Antibodies

Electrochemical immunosensors require the immobilization of antibodies on their surface in order to detect a particular analyte, hence this process is essential to the creation of the sensor. Activating the sensor surface and crosslinking the antibodies to the surface are typically the two basic processes in the process of immobilizing antibodies on the surface of an electrochemical immunosensor. Activating the sensor surface is the initial step in immobilizing antibodies on the surface. This is commonly accomplished by creating reactive groups, such as carboxylic acid or amine groups, to the sensor's surface, which can then be utilized to covalently attach the antibodies to the surface. Chemical or physical techniques, such UV radiation or plasma treatment, can be used to activate the surface. The antibodies can be crosslinked to the surface once it has been activated. Several techniques, including glutaraldehyde crosslinking, EDC crosslinking, protein A crosslinking, and Au-S bond

formation, can be employed to crosslink the antibodies to the surface. These techniques rely on the formation of covalent or noncovalent bonds through chemical or physical interactions between the reactive groups on the sensor surface and the functional groups on the antibodies (Dinckaya *et al.*, 2011).

Covalent attachment of antibodies to a gold surface can be achieved using the frequently used crosslinking agent glutaraldehyde. Normally, the gold surface is activated first, and then antibodies are incubated with the activated gold surface in the presence of glutaraldehyde, forming covalent bonds between the antibodies and the gold surface.

It's vital to remember that the type of sensor and the kind of antibodies being employed will determine the precise procedures and circumstances needed to activate and crosslink the antibodies. To get the desired specificity and stability of the immobilized antibodies, the conditions and reagent ratio may need to be optimized.

1.5.3 Blocking of the Sensor Surface

Blocking in the context of immunosensor development is the technique of preventing analytes or impurities from adhering non-specifically to the sensor's surface. Because it can result in false positive or false negative readings, non-specific binding can compromise the immunosensor's precision and sensitivity. An immunosensor surface can be blocked using a variety of methods, including chemical blocking and physical blocking. Chemical blocking involves applying a chemical to the sensor's surface to coat it and prevent non-specific binding, such as a protein or carbohydrate. Physical blocking includes separating the sensor surface from the sample by a physical barrier, like a membrane or filter. Blocking is a crucial stage in the development of immunosensors because it reduces non-specific binding, which increases the sensor's specificity and sensitivity. Blocking can also help to increase the sensor's shelf life by preventing surface degradation or contamination. Overall, blocking is an important phase in the development of immunosensors, and the choice of blocking technique will rely on the particular needs and specifications of the sensor.

A substance called ethanolamine is frequently employed as a blocking agent in the creation of immunosensors. A blocking agent is a chemical that is used to inhibit non-specific binding of

impurities or analytes to the surface of the sensor, which can affect the immunosensor's sensitivity and accuracy. Ethanolamine functions as a blocking agent by obstructing the sensor's surface and preventing the non-specific binding of impurities or analytes. An aqueous solution of ethanolamine can create a stable monolayer on the surface of the sensor because it is a polar molecule. This monolayer functions as a barrier to stop contaminants or analytes from non-specifically adhering to the sensor's surface.

Ethanolamine is a blocking agent that can also be used to stabilize the sensor's surface and ward off deterioration or contamination. Additionally, it can be used to modify the pH of the sensor surface, which may be crucial for enhancing the sensor's functionality.

Overall, ethanolamine is an effective blocking agent for the construction of immunosensors because it can block the sensor's surface, obstruct non-specific binding, stabilize and shield the sensor surface.

1.6 Biomarkers for diagnostic applications

1.6.1 IKZF1 and IKZF3

The transcription factors IKZF1 (Ikaros family zinc finger 1) and IKZF3 (Ikaros family zinc finger 3) are encoded by the *IKZF1* and *IKZF3* genes, respectively. These proteins serve crucial roles in the growth and operation of immune cells such T cells and B cells, as well as in the regulation of gene expression. Multiple myeloma, a form of cancer that affects plasma cells in the bone marrow, is treated with the chemotherapeutic drug lenalidomide. Lenalidomide functions by inhibiting the formation of immune cells, such as T cells and B cells.

IKZF1 and IKZF3 are related to lenalidomide in that lenalidomide can influence the expression of these genes. Lenalidomide, in particular, has been demonstrated to downregulate IKZF1 and IKZF3 expression in T cells and B cells, which may contribute to its immune-suppressive effects. Lenalidomide's mechanism of action may also involve IKZF1 and IKZF3, as their downregulation has been associated with the prevention of tumor growth and angiogenesis in multiple myeloma (Pourabdollah *et al.*, 2016). IKZF1 and IKZF3 are transcription factors that are crucial for the growth and operation of immune cells, and lenalidomide is a chemotherapy treatment that can alter the expression of these genes and potentially inhibit the immune system.

1.6.2 Lysosomal Storage Disorders (LSDs)

Lysosomal storage disorders are a class of inherited metabolic diorders caused by lysosomal enzyme deficiencies. Small organelles called lysosomes are present inside cells and are in charge of digesting and recycling numerous biomolecules, including proteins, lipids, and carbohydrates. They contain a range of hydrolytic enzymes designed specifically to digest various classes of biomolecules.

A buildup of biomolecules within the lysosomes occurs in people with lysosomal storage diseases because one or more of these lysosomal enzymes is absent or isn't working properly. This buildup of biomolecules has the potential to damage tissues and cells, resulting in a wide spectrum of symptoms that may impact different organ systems.

More than 50 different lysosomal storage disorders exist, and many different lysosomal enzyme deficiencies can lead to them. Gaucher disease, Niemann-Pick disease, Tay-Sachs disease, Pompe disease, Gaucher disease, Krabbe disease, and Fabry disease are a few examples of lysosomal storage disorders. These conditions can be inherited in various ways, including X-linked inheritance and autosomal recessive inheritance, and they can be caused by mutations in a wide range of genes.

Pompe disease, Gaucher disease, and Krabbe disease are all examples of lysosomal storage disorders. These disorders are caused by defects in specific lysosomal enzymes, which leads to the accumulation of biomolecules within lysosomes. This accumulation of biomolecules can cause damage to cells and tissues, leading to a wide range of symptoms that can affect multiple organ systems.

Pompe disease is an inherited metabolic disorder that is caused by a deficiency in the enzyme acid α-glucosidase (GAA). This enzyme is responsible for breaking down glycogen, a complex carbohydrate that is stored in the muscles and liver. In individuals with Pompe disease, glycogen accumulates in the cells, leading to muscle weakness and damage, as well as other symptoms. Pompe disease can be severe and life-threatening, and it often requires treatment with enzyme replacement therapy.

Gaucher disease is an inherited metabolic disorder that is caused by a deficiency in the enzyme β-Glucocerebrosidase (GBA). This enzyme is responsible for breaking down a specific lipid called glucocerebroside. In individuals with Gaucher disease, glucocerebroside accumulates in the cells, leading to symptoms such as anemia, bruising, and organ enlargement. Gaucher disease can be treated with enzyme replacement therapy, as well as other supportive measures.

A lack of the enzyme galactosylceramidase, commonly known as galactocerebrosidase (GALC), results in Krabbe disease (KD), an inherited metabolic disorder. This enzyme is in charge of degrading a particular lipid known as galactosylceramide. Galactosylceramide builds up in the cells of people with Krabbe disease, causing damage to the myelin sheath, the covering that protects nerve cells. This may result in neurological symptoms like visual loss, developmental delays, and muscle weakness.

Enzyme replacement therapy or other supportive care is frequently used to treat lysosomal storage diseases since they can be serious and life-threatening. The prognosis for those who have these conditions can be improved with early identification and treatment.

1.6.3 Dengue Virus (NS1 antigen)

Dengue virus is a mosquito-borne virus that is responsible for the disease dengue fever. It is a single-stranded RNA virus that is spread to people by the Aedes mosquito and is a member of the Flaviviridae family. There are four different serotypes of the dengue virus (DENV-1, DENV-2, DENV-3, and DENV-4), and exposure to one of them confers immunity to that serotype but not to the other three. Viral dengue fever is characterized by a fever, headache, pain in the muscles and joints, and a rash. Dengue hemorrhagic fever, which can be fatal, can develop from dengue fever in severe cases. The nonstructural protein 1 (NS1) is one of the antigens that the dengue virus generates. Early in the course of the infection, a glycoprotein called NS1 is created, and it's thought to help the virus evade the immune system. NS1 is present in the blood of infected individuals, and it has been used as a diagnostic marker for dengue infection. For the purpose of identifying dengue virus infection, various commercially available immunoassays utilise the target protein NS1.

NS1 is being investigated as a potential target for the development of a dengue vaccine in addition to its usage as a diagnostic marker. Given that NS1 is highly conserved throughout the four dengue virus serotypes and is known to trigger an effective immune response, researchers are looking into the possibility of employing it as a subunit vaccine. A glycoprotein called nonstructural protein 1 (NS1) is created by the dengue virus when it is infected. It has been utilized as a diagnostic indicator for dengue infection since it can be found in the blood of those who have the disease (Nunes *et al.*, 2018).

NS1 is a helpful diagnostic sign because it is created early in the course of the illness and can be found in the blood before other diagnostic indicators, including dengue virus antibodies, can be seen. As a result, NS1 can be used to diagnose dengue disease early on and to distinguish it from other viral diseases that may have symptoms that are identical (Mishra *et al.*, 2019).

NS1 is a target for several commercially available immunoassays that look for dengue virus infection. These tests can be conducted on serum or blood samples and typically utilize the use of an immobilized NS1-specific antibody on a solid support, like a microplate. The sample is added to the solid support, and if NS1 is present in the sample, it will bind to the immobilized antibody. An electrical current or a colorimetric indicator can be used to identify and measure this binding event.

Overall, NS1 is a helpful biomarker for dengue virus infection due to its early production during the course of the infection, ease of blood detection, and dengue virus-specificity. This makes NS1 a valuable tool for the diagnosis and management of dengue infection.

CHAPTER 2

SCIENTIFIC ARTICLE

DEVELOPMENT OF LABEL-FREE IMPEDIMETRIC IMMUNOSENSORS FOR IKZF1 AND IKZF3 FEMTOMOLAR DETECTION FOR MONITORING MULTIPLE MYELOMA PATIENTS TREATED WITH LENALIDOMIDE

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CONTRIBUTION OF THE MAIN AUTHOR AND CO-AUTHORS

Haya Abdulkarim and Prof. Mohamed Siaj conceived the idea of this work.

Haya Abdulkarim designed and performed all the experiments, analyzed the data and wrote the full manuscript.

Prof. Mohammed Zourob has critically reviewed the idea of the work and was involved in discussions during the project.

All authors have read, given feedback and approved the current version of the manuscript for publication.

2.1 Abstract

Lenalidomide is an immunomodulatory drug (IMiD) used to treat multiple myeloma (MM) patients. Lenalidomide destroys MM cells by inducing ubiquitination and the consequent degradation of Ikaros family zinc finger proteins 1 and 3 (IKZF1 and IKZF3). High expression of IKZF1 and IKZF3 in MM results in less sensitivity to lenalidomide treatment and possible cytotoxic effect. Therefore, detecting the expression of IKZF1 and IKZF3 proteins is of utmost importance in the treatment of MM. Here, we report the fabrication of a novel label-free electrochemical immunosensor for the rapid detection and quantification of IKZF1 and IKZF3 using electrochemical impedance spectroscopy (EIS). Gold electrodes were used to fabricate the immunosensors by immobilizing IKZF1 and IKZF3 specific antibodies using cysteamine and PDITC (*p*-Phenylene diisothiocyanate) crosslinkers. The immunosensors were able to detect IKZF1 and IKZF3 protein levels with respective low detection limits of 0.68 and 0.97 pg/ml (11.8 and 16.7 fM). Furthermore, the immunosensors' successful application in human serum and their high selectivity and sensitivity enables their possible application in other biofluids as simple point-of-care devices for monitoring multiple myeloma patients treated with lenalidomide, to prevent the drug's cytotoxicity and minimize its side effects.

2.2 Introduction

Multiple myeloma (MM) is the most common type of plasma cells cancer. It's responsible for around tenth of all the haematological tumors, and 1.6 % of all cancer cases (Michels et Petersen, 2017). Multiple myeloma is characterized by the irregular increase in the clonal plasma cells leading to the organ dysfunction (Al-Farsi, 2013). Some of the symptoms associated with MM are nausea, weakness, and vomiting, others only manifest laboratory abnormalities like anemia and high monoclonal protein levels in serum or urine, and elevated clonal plasma cells in the bone marrow (Al-Farsi, 2013; Michels et Petersen, 2017). Multiple myeloma is known to be incurable, but major improvements happened in recent years, allowing younger patients to have a higher median survival life span (Al-Farsi, 2013).

Generally, the discovery of immunomodulatory drugs (IMiDs) like Lenalidomide and Thalidomide, introduced some treatment options for MM (Al-Farsi, 2013; Awwad *et al.*, 2018). However, Thalidomide was first used as a sedative and later for the treatment of MM, but then

proven to cause miscarriages and birth defects, resulting in discontinuing its use (Goldman, 2001). A new less toxic IMiD called Lenalidomide (Revlimid) was later discovered (Al-Farsi, 2013), and was proven to be a highly effective treatment for MM (Fink et Ebert, 2015). Lenalidomide's minimal neurotoxicity allows it to be a long-term administrable drug for multiple myeloma patients (Dimopoulos *et al.*, 2013).

In multiple myeloma, the continuous expression of Ikaros family zinc finger protein 1 (IKZF1) and Ikaros family zinc finger protein 3 (IKZF3) is essential for the proliferation and survival of the myeloma cells (Fink et Ebert, 2015). IKZF1 and IKZF3 are transcriptional factors that are important in the differentiation of lymphocytes, but their post transcriptional regulation is still poorly studied (Awwad *et al.*, 2018). Lenalidomide kills multiple myeloma cells by inducing the IKZF1 and IKZF3 ubiquitination, causing the proteasomal degradation of both IKZF1 and IKZF3 B-cell transcription factors (Fink et Ebert, 2015). Krönke *et al*. (Krönke *et al.*, 2017) suggested that the high expression of IKZF1 and IKZF3 in MM cells resulted in less sensitivity to lenalidomide treatment. The study also described the selective degradation of IKZF1 and IKZF3 caused by Lenalidomide in MM, and the importance of their loss during the therapeutic activity of Lenalidomide (Krönke *et al.*, 2014; Lu *et al.*, 2014). Several hours after treatment with lenalidomide, IKZF1 and IKZF3 get down regulated and degraded rapidly (Zhu *et al.*, 2014).

Several immunoassays have been employed previously, including immunohistochemistry technique which was used to quantify IKZF1 and IKZF3, but it only permitted the semi-quantification of proteins and interpretation of the results require experienced pathologists (Misiewicz-Krzeminska *et al.*, 2018). Flow cytometry was also used (Zhu *et al.*, 2014) for the analysis of IKZF1, as well as immunoblotting for IKZF1 and IKZF3 after the treatment with lenalidomide for MM patients (Zhu *et al.*, 2014). Enzyme-linked immunosorbent assay (ELISA) and western blot (WB) (Misiewicz-Krzeminska *et al.*, 2018) are amongst other methods used. However both are time consuming, involve labeling with enzymes or tags (Eissa, S., Alshehri, N., *et al.*, 2018), and require trained staff and centralized labs to be performed (Elshafey, R. *et al.*, 2013) and relatively large sample quantities.

Nowadays, more research is being conducted on simpler, more rapid, and cost-effective analytical assays such as biosensors and lab-on-a-chip (LOC) devices. More specifically, electrochemical biosensors show great promise due to their significant lower cost, minimization of the sample volume used, and their high throughput screening (Eissa *et al.*, 2015), therefore they're applicable as point-of-care testing (POCT) devices. To the best of our knowledge, this is the first report for the development of electrochemical biosensors for the detection and quantification of IKZF1 and IKZF3. Therefore, in this work we report the fabrication of new label-free impedimetric immunosensors for the detection of IKZF1 and IKZF3 at the femtomolar levels for MM patients given lenalidomide treatment. The immunosensors were fabricated by immobilizing IKZF1 and IKZF3 specific antibodies on classic gold electrodes and testing them further with different concentrations of the proteins. The method used was electrochemical impedance spectroscopy (EIS) which is known to be a non-destructive technique done in the presence of a redox couple also referred to as faradaic impedance measurements (Eissa, S., Abdulkarim, Haya, *et al.*, 2018; Elshafey, R. *et al.*, 2013). The detection was achieved by measuring the change in the charge transfer resistance as proteins bind to their respective immobilized antibodies. These label-free impedimetric immunosensors can be possibly useful for monitoring the response to lenalidomide in MM patients by detecting IKZF1 and IKZF3, and maybe later applied for monitoring different immunomodulators or therapeutic agents.

2.3 Experimental

2.3.1 Materials and reagents

Cysteamine hydrochloride, Sulfuric acid, 1,4-phenylene diisothiocyanate (PDITC), pyridine, potassium ferrocyanide ($K_4Fe(CN)_6$), potassium ferricyanide ($K_3Fe(CN)_6$), ethanolamine, sodium chloride, N-N dimethylformamide (DMF), hydrogen peroxide, acetonitrile, ethanol, bovine serum albumin (BSA), phosphate buffered saline (PBS pH 7.4) tablets, and human serum (from male AB clotted whole blood) were obtained from Sigma-Aldrich (St. Louis, MO, USA). MicroPolish alumina 0.05, 0.3, and 1.0 µm and polishing pads were bought from Buehler (Lake Bluff, IL, USA). Human DNA-binding Protein Ikaros (IKZF1) ELISA kit, and Human Zinc Finger Protein Aiolos (IKZF3) ELISA kit were purchased from Mybiosource (San Diego, CA, USA). PBS (pH 8.5) was used for the preparation of IKZF1 and IKZF3 antibody

solutions. Highly pure water was obtained after Milli-Q plus treatment (Millipore, Billerica, MA, USA) then used for the preparation of solutions.

2.3.2 Instrumentation

All electrochemical measurements for both cyclic voltammetry (CV) and Electrochemical Impedance Spectroscopy (EIS) were done using AUTOLAB PGSTAT302N potentiostat (Metrohm, Netherlands). The potentiostat contains a frequency response analyzer (FRA) connected to a computer run by NOVA 1.9 software. A classical electrochemical cell setup with three electrodes (counter, reference, and working) was used in all electrochemical experiments. The reference electrode is an Ag/AgCl (1M KCl), the counter electrode is a platinum wire, and the working electrode is a standard gold electrode.

2.3.3 Methods

2.3.3.1 Fabrication of IKZF1 and IKZF3 immunosensors

The surface of bare gold working electrodes was first polished until a mirror finish was obtained using 1.0, 0.3 and 0.05 μm alumina slurries on polishing pads for 5 minutes each. Polished electrodes were then cleaned with piranha solution 1:3 v/v of hydrogen peroxide (H_2O_2) and concentrated sulfuric acid (H_2SO_4), respectively, for 2 minutes at room temperature, then rinsed with deionized water followed by sonication in absolute ethanol for 2 minutes. The gold electrodes were then electrochemically cleaned by cycling the potential (vs. Ag/AgCl) between -0.2 and 1.6 V at 100 mV/s in 0.1 M H_2SO_4 for 30 cycles, then washed with deionized water. Subsequently, self-assembled monolayers were formed on the electrode surfaces, by incubating them in 10 mM cysteamine hydrochloride for 2 hours in room temperature. The modified electrodes were then washed with absolute ethanol to eliminate excess cysteamine, then incubated in 10 mM PDITC solution for 2 hours. PDITC was prepared as a mixture of 1:9 v/v pyridine and DMF. Electrodes were later washed with DMF and ethanol extensively. The modified electrodes were then incubated in a solution of PBS (pH 8.5) containing 1 μg/ml of IKZF1 or IKZF3 antibodies for 2 hours at room temperature, then washed with PBS (pH 7.4) to eliminate unbound antibodies from the surface. Finally, the electrodes were blocked by incubating them for 30 minutes in 0.1 M ethanolamine. They were

washed with PBS (pH 7.4) afterwards, and directly used for detecting IKZF1 and IKZF3, or stored in PBS at 4˚C.

2.3.3.2 Immunosensor detection experiments

Electrodes with immobilized IKZF1 and IKZF3 were incubated for 20 minutes in tubes containing 100 µL of IKZF1 and IKZF3 protein solutions diluted in PBS (pH 7.4) at different concentrations (from 16 pg/ml to 1 ng/ml), electrodes were then washed with PBS buffer after each incubation. EIS and CV were the electrochemical measurements utilized for detecting.

2.3.3.3 Electrochemical measurements

5 mM of Ferri/Ferrocyanide in PBS (pH 7.4) solution was used in all electrochemical measurements for EIS and CV. CV measurements were done by cycling the potential between -0.2 to 0.5 V at a scan rate of 100 mV/s. Whereas EIS measurements were recorded at a frequency of 10 kHz to 0.1 Hz, AC voltage of 10 mV and DC potential of 0.2 V. Nova 1.9 was used to fit all Impedance data displayed as Nyquist plots.

2.3.3.4 Selectivity experiments

IKZF1 and IKZF3 immunosensors were tested for cross reactivity between the two proteins and for Bovine serum albumin (BSA). IKZF1 immunosensors were incubated in 100 µL of 0.5 ng/ml of IKZF3 or 1% BSA in PBS (pH 7.4). Similarly, IKZF3 immunosensors were incubated in IKZF1 and BSA for 20 minutes at room temperature.

2.3.3.5 Application of the immunosensors in spiked serum samples

The immunosensors were applied on spiked serum samples, in order to examine the applicability of the sensor in real biological samples. Human serum samples (Sigma-Aldrich) were diluted 1:8 in PBS buffer (pH 7.4) and spiked with 0.125, 0.25 and 0.5 ng/ml of IKZF1 or IKZF3, then incubated on the electrode surface for 20 minutes at room temperature. Electrodes were then washed with PBS buffer and measured.

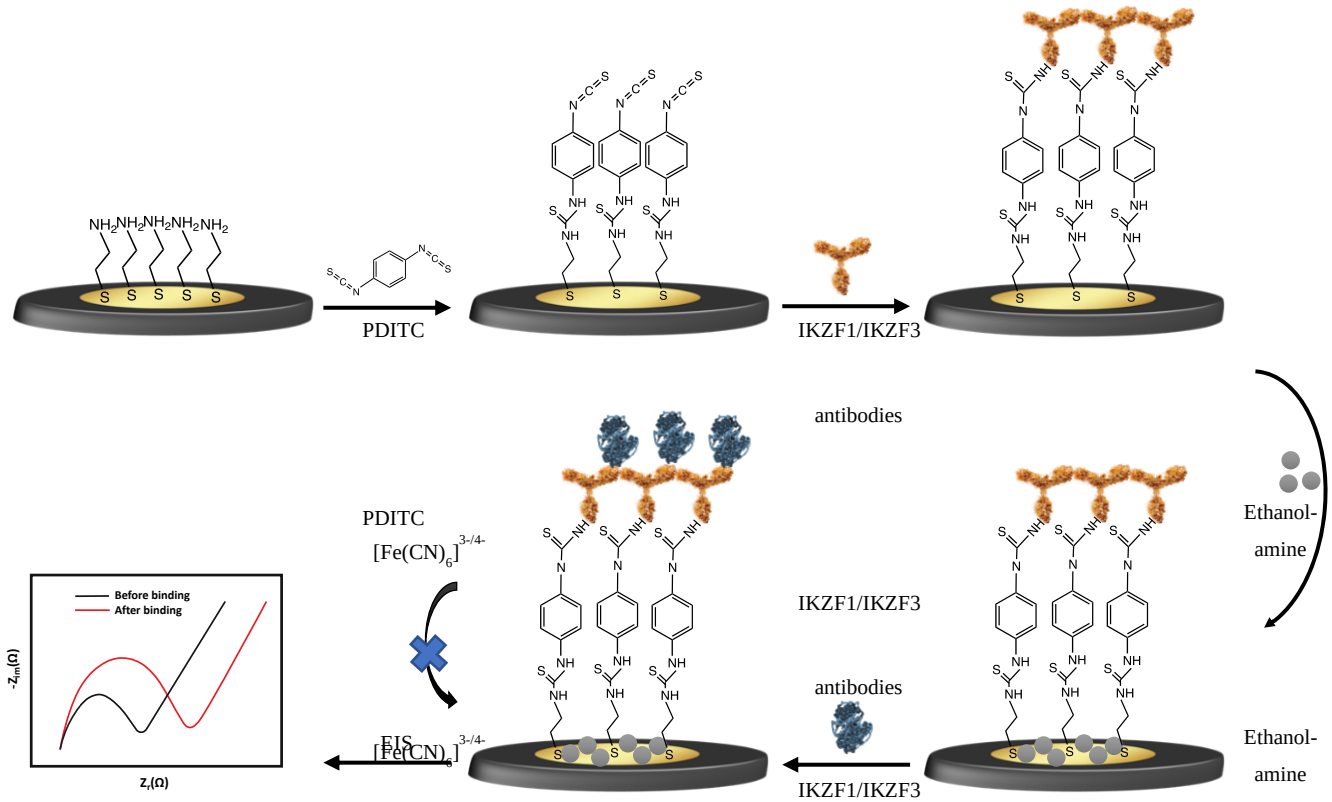
2.3.3.6 ELISA experiments for IKZF1 and IKZF3 in serum

Duplicates of ELISA for IKZF1 and IKZF3 were performed according to the manufacturer's protocol. First, the standards and spiked diluted serum samples were added to the antibody precoated wells and incubated for 2 hours at 37 °C. After washing, 100 μL of Biotin-conjugated secondary antibodies for 1 hour at 37 °C. Wells were washed and incubated for 1 hour with 100 μL of Horseradish Peroxidase (HRP) conjugated avidin at 37 °C. 90 μL of 3,3',5,5'-Tetramethyl-benzidine (TMB) substrate solution were added to the wells and incubated for 15-30 minutes, were HRP will reduce hydrogen peroxide and oxidize TMB, the reaction is then stopped by adding 50 μL of sulfuric acid stop solution. Readings were taken at 450 nm using a microplate reader.

2.4 Results and Discussion

2.4.1 Fabrication of the IKZF1& IKZF3 immunosensors

The immunosensors were fabricated by the immobilization of IKZF1 and IKZF3 specific antibodies on the surface of gold electrodes (Scheme 2.1). For immobilization, standard gold electrodes were first modified with cysteamine and PDITC as a linker. Cysteamine was first incubated on the surface to form a self-assembled monolayer (SAM) containing terminal amine groups. PDITC was then used on cysteamine modified electrodes as a cross-linker. The formation of the thiourea bonds allows of the thiocyanate groups from the PDITC to react with amine groups of the antibodies, and the other thiocyanate group would react with the amine groups present on the gold surface (Eissa, S., Abdulkarim, Haya, *et al.*, 2018). Finally, ethanolamine was used to block the unreactive thiocyanate groups and minimize the non-specific adsorption that might generate false signals when later applying the immunosensor. The immunosensor's stability was also tested after storing it in PBS buffer pH 7.4 in 4°C for 2 weeks with a response change of 2.8% and 1.9% for IKZF1 and IKZF3 immunosensors, respectively.



Scheme 2.1 Schematic diagram of the fabrication steps and detection of the IKZF1 and IKZF3 immunosensors.

2.4.2 Electrochemical characterization of the immunosensor

EIS and CV were used to electrochemically characterize the gold electrode surface for each step of the functionalization and of the IKZF1 and IKZF3 immunosensors. Both were recorded for each fabrication step of the two immunosensors in ferro/ferricyanide redox couple solution. For CV (Fig 2.1a.), a sharp reversible peak was first observed for the clean gold electrode, exhibiting a peak-to-peak separation (ΔE). After functionalization with cysteamine, there was a slight decrease in the ΔE indicating the enhancement of the electron transfer due to the positive charge of the amine groups. But the addition of the PDITC and its reaction with cysteamine caused an increase in ΔE as the peak current decreased, that's attributed to the negative charge of the composed terminal isothiocyanate groups which inhibited the electron

transfer by revolting the redox anions (Eissa, S., Abdulkarim, Haya, *et al.*, 2018). The immobilization of the antibodies caused the ΔE to further increase and the electron transfer to decrease due to the blockage caused by the bulky sized antibodies.

For each fabrication step, EIS was measured as shown in (Figs 2.1b-2.1c.). Nyquist plots were obtained and fitted using modified Randles equivalent circuit consisting of solution resistance (R_S), charge transfer resistance (R_{ct}), constant phase element (CPE), and Warburg impedance (Z_W). Nyquist plots obtained for each step consist of a semicircle indicating the charge transfer process, followed by a straight line indicating the diffusion of redox molecules. For the bare gold electrode, a typical small semicircle with a straight line was observed. After the functionalization of the surface with cysteamine, the semicircle diminished indicating a lower charge transfer resistance. After the functionalization with the crosslinker PDITC and the immobilization of the antibodies, the semicircle broadened significantly implying the slower electron transfer rate and higher electron transfer resistance. The semicircle will only further increase as each immunosensor is incubated with a higher concentration of its respective proteins, due to the proteins' large size and their increasing blockage of the electrode surface, demonstrating the fundament of this immunosensor's detection method.

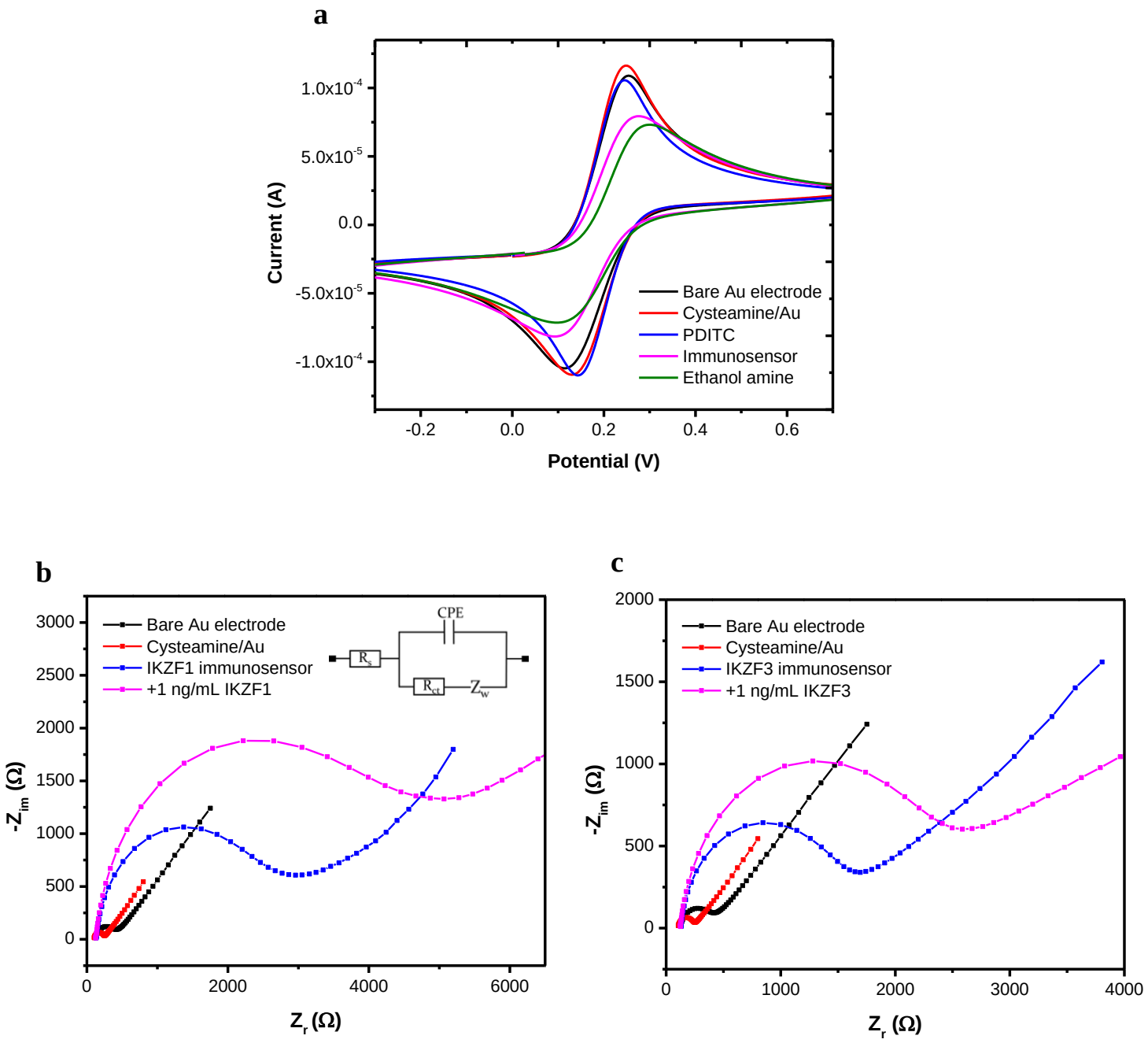


Figure 2.1 Cyclic voltammetry peaks for the characterization of the fabrication step of the immunosensors (a). Nyquist plots for the fabrication of IKZF1 immunosensor (b) and IKZF3 immunosensor (c). Figure 2b shows the modified Randles equivalent circuit used to fit all the EIS Nyquist plots in this work. CV was measured at scan rate of 100 mV/s, and EIS measurements were all done at 10 KHz to 0.1 Hz frequency range. Electrochemical measurements were done in ferro/ferricyanide redox couple solution in PBS pH 7.4.

2.4.3 Binding experiments of the immunosensors with their specific proteins and binding time optimization

The binding time of antibodies to their antigens is important to ensure the highest signal response in the shortest time. In order to optimize this parameter for IKZF1 and IKZF3 immunosensors, both were incubated with 0.25 ng/ml of their respective analytes diluted in PBS (pH 7.4), then EIS plots were obtained at different periods (from 5 to 30 minutes). The percentage of change in the resistance before and after binding was used to calculate the sensor response as the following $((R-R^o)/R^o\ \%)$, where R represents the resistance of the sensor after binding, and R^o is the resistance before binding. Figs 2.2a-2.2b show the responses of IKZF1 and IKZF3 immunosensors at different times. It is clear that the response was increasing until 20 minutes, after which no apparent increase was shown. The highest signal was obtained for IKZF1 immunosensor at 20 minutes, and between 15-20 minutes for IKZF3 immunosensor, indicating that 20 minutes is the shortest and optimal time to obtain the highest binding signal.

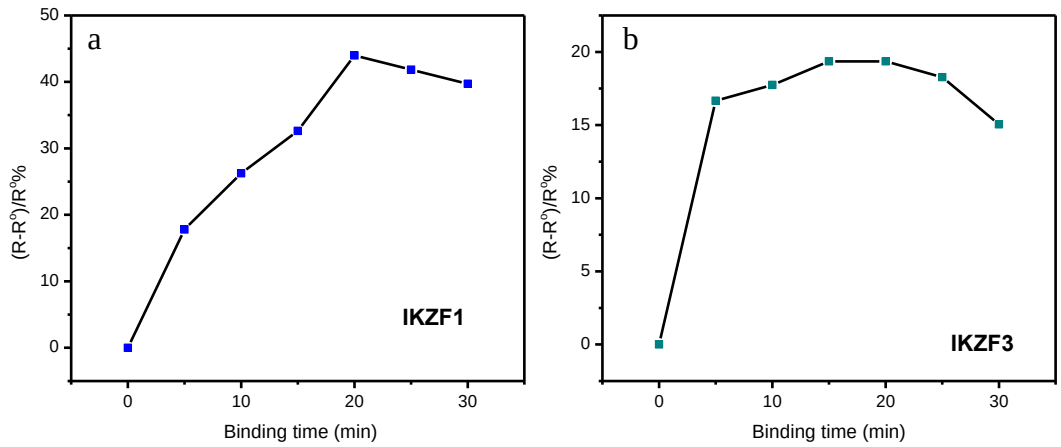


Figure 2.2 Plot of the immunosensor response versus the binding time in minutes of the immunosensors with their specific proteins for IKZF1 (a) and IKZF3 (b).

2.4.4 Dose-response of IKZF1 & IKZF3 Immunosensors

The immunosensors were incubated in different concentrations ranging from 16 pg/ml to 1 ng/ml for both IKZF1 and IKZF3, and EIS was recorded for each concentration to test the analytical range of response. In (Fig 2.3a-2.3b), Nyquist plots are shown for both immunosensors, for concentrations: 0, 0.016, 0.031, 0.063, 0.125, 0.25, 0.5, and 1 ng/ml. The consistent enlargement of the semicircle diameter is due to the increase of the charge transfer resistance caused by the increased concentration of proteins binding to the immunosensor. The data for both immunosensors were plotted into two calibration curves as shown in (Fig 2.3c-2.3d), where the log of the protein concentration was plotted against the immunosensor response signal.

The linear regression equations for the immunosensors were: $(R-R^o)/R^o$ % = 74.4 + 43.5 log C [ng/ml], R=0.997 for IKZF1, and $(R-R^o)/R^o$ % = 60.4 + 30.6 log C [ng/ml], R=0.985 for IKZF3. To confirm the high accuracy of the immunosensors, EIS plots were taken several times and the standard deviations of measurements were demonstrated as error bars (RSD% < 4%). Both curves showed a linear response from 16 pg/ml to 1 ng/ml, and LOD were calculated to be 0.68 pg/ml (11.8 fM) for IKZF1, and 0.97 pg/ml (16.7 fM) for IKZF3 respectively, signifying the high sensitivity of the sensors at fM levels. However, the LOD of the ELISA kits were reported to be 31.25 pg/ml and 25 pg/ml for IKZF1 and IKZF3, respectively. ELISA has an Inter-Assay and Intra-Assay CV% for kits of < 8 % and < 10 %, respectively, as indicated by the manufacturer, whereas our sensors indicated < 5 % and < 3 % showing better reproducibility. The fabricated sensors are shown to be superior to ELISA by being label-free, cost-effective, faster, and have high potential to be applied as a point-of-care near bead-site or in-field applications.

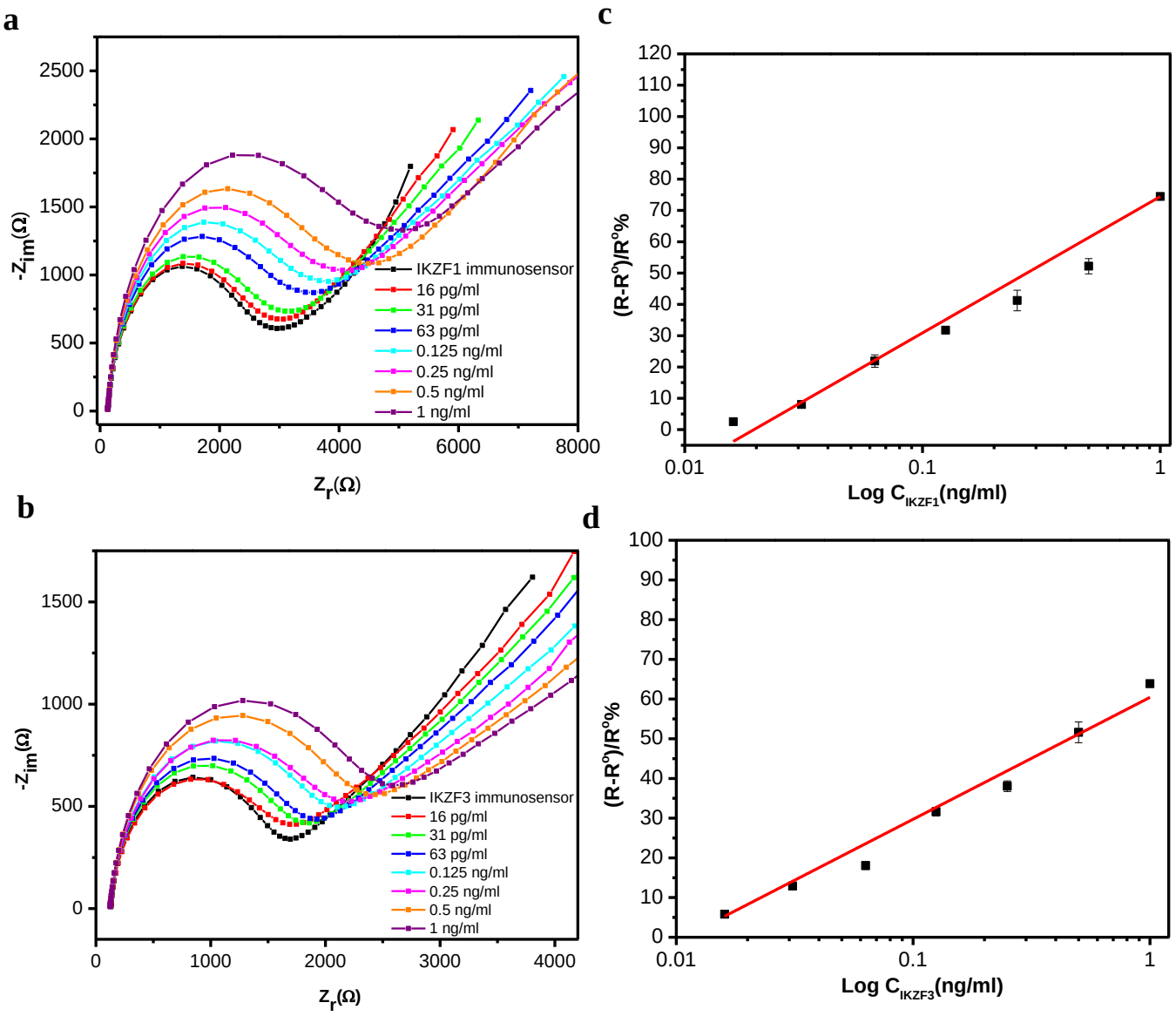


Figure 2.3 Plot of the immunosensor response versus the binding time in minutes of the immunosensors with their specific proteins for IKZF1 (a) and IKZF3 (b). EIS Nyquist plots after binding with different concentrations of IKZF1 (a) and IKZF3 (b), and the calibrations curves for IKZF1 (c) and IKZF3 (d) plotting the logarithm of concentrations in ng/ml versus the immunosensor's response. Error bars were calculated as the SD of measurements.

2.4.5 Cross reactivity testing of IKZF1 & IKZF3 immunosensors

The selectivity of the immunosensors towards their specific proteins were examined against the other protein and against BSA. IKZF1 immunosensor was incubated in 0.5 ng/ml IKZF3 proteins and BSA, while IKZF3 immunosensor was incubated in 0.5 ng/ml IKZF1 protein and BSA. A drastic difference between the response of the sensors was observed towards their specific analyte in comparison with the non-specific proteins and BSA. Figs 2.4a and 2.4b show that IKZF1 immunosensor indicated a 52.18 % response corresponding to its specific protein, in comparison to 1.27 % response to IKZF3 proteins and 1.59 % for BSA. On the other hand, IKZF3 immunosensor showed a 51.61 % response corresponding to its specific protein, in comparison to 1.25 % response to IKZF1 proteins and 1.36 % for BSA. The results above demonstrate the high selectivity and very low cross reactivity of the developed immunosensors.

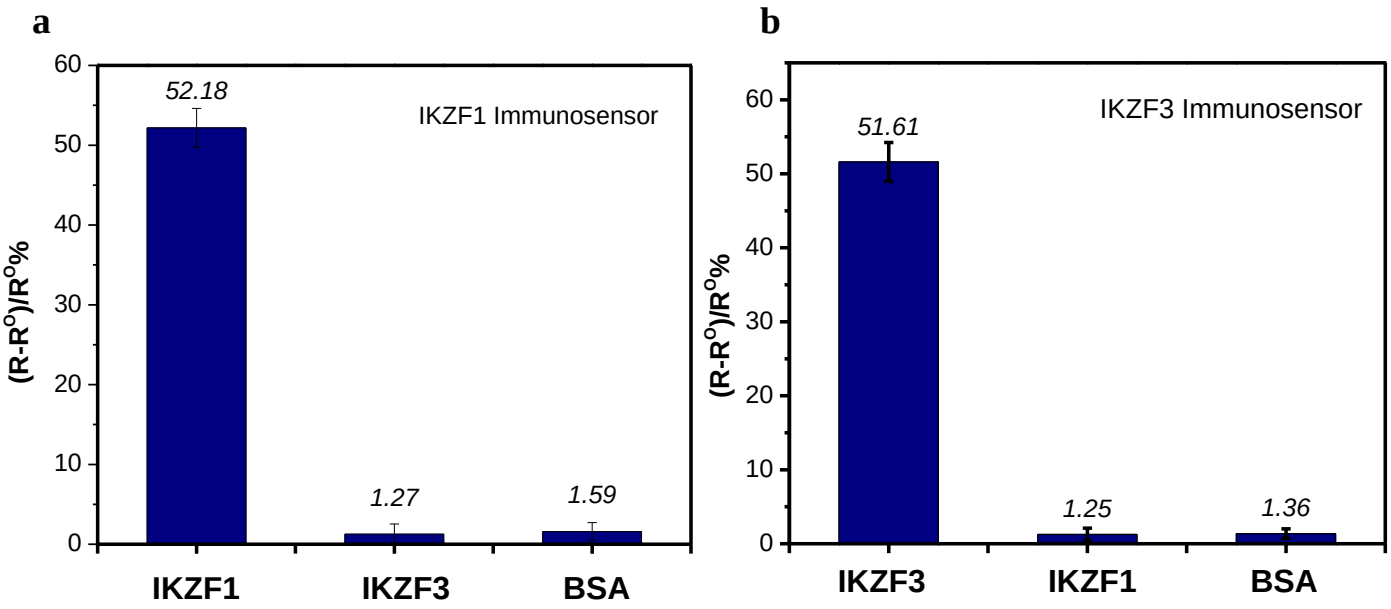


Figure 2.4 Cross reactivity experiments for the response of IKZF1 immunosensor against 0.5 ng/ml IKZF3 and 1% BSA (a), and IKZF3 immunosensor's response against 0.5 ng/ml IKZF1 and 1% BSA (b), all in PBS buffer pH 7.4.

Table 2.1 Two human serum samples (diluted 1:8) one spiked with 0.5 ng/ml of IKZF1 and the other with 0.5 ng/ml IKZF3, recovery %, and the relative standard deviation of duplicate measurements.

	Spiked concentrations *(pg/ml)*	Measured concentrations *(pg/ml)*	Recovery (%)	RSD (%)
IKZF1	500	482.3	96.5	13.2
IKZF3	500	506	101.2	2.8

2.4.6 Initial application of immunosensors in spiked serum samples

To test the immunosensors and their ability to be applied in biological samples, they were applied on 1:8 diluted human serum spiked with 0.5 ng/ml IKZF1 and IKZF3. After measuring the concentrations, the relative standard deviation (RSD %) was calculated for the two measurements (**Table 2.1.**). To further confirm the range of linearity, two more concentrations were tested by spiking serum with 0.125 and 0.25 ng/ml and plotting them against the measured concentrations (**Fig 2.5a.**). Spiked concentrations were then plotted against their recovery % that ranges between 92.6 % and 101.2 % for IKZF1/3 at different concentrations (**Fig 2.5b.**). The resulting recovery % eliminate the notable serum matrix effect on the immunosensor, indicating the sensors' good precision and possible applicability in other biological samples suitable for IKZF1/3 detection.

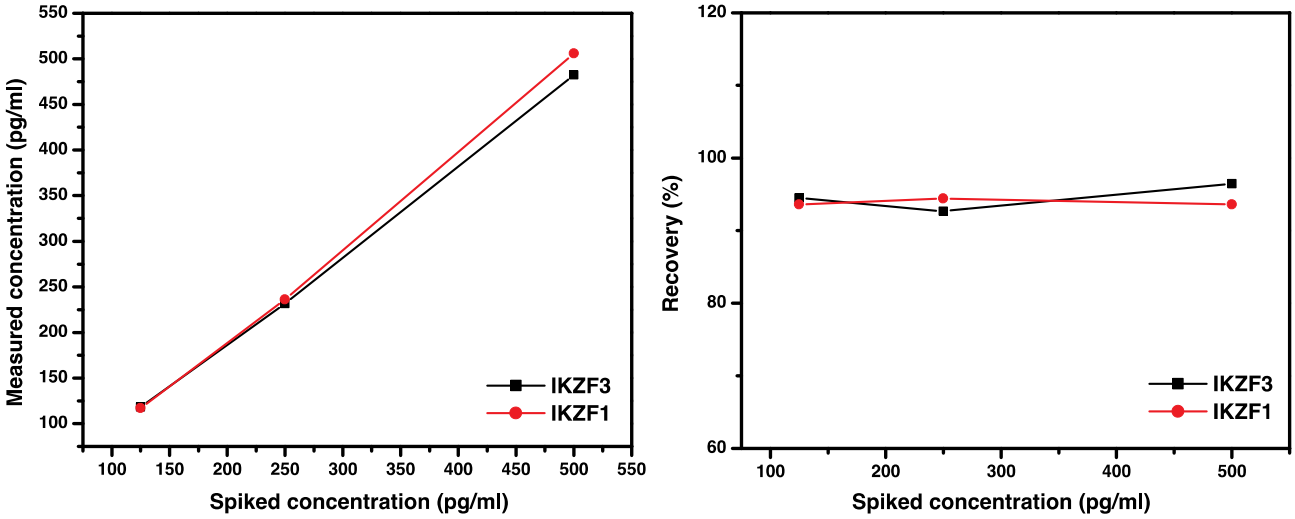


Figure 2.5 Spiked serum samples of IKZF1 and IKZF3 of different concentration 125, 250 and 500 pg/ml were plotted against the measured concentrations of the samples (a) their Recovery % (b).

2.5 Conclusion

To summarize, new and simple label-free impedimetric immunosensors for the detection and quantification of IKZF1 and IKZF3 were fabricated. The rapid immunosensors showed very good sensitivity and selectivity against the other proteins, as well as good LOD of 11.8 and 16.7 fM (0.68 pg/ml and 0.97 pg/ml) for IKZF1 and IKZF3 immunosensors, respectively. The electrochemical immunosensors' successful application in human serum opens the possibility of their future application in other biological samples taken from patients with MM treated with lenalidomide. Those biosensors could possibly work as point-of-care devices to monitor patient's response and resistance to lenalidomide and treatment dosages required, to perhaps prevent cytotoxicity and reduce its side effects.

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CHAPTER 3

SCIENTIFIC ARTICLE

LABEL-FREE IMMUNOSENSOR FOR THE MULTIPLEXED DETECTION OF ACID α-GLUCOSIDASE, β-GLUCOCEREBROSIDASE AND GALACTOCEREBROSIDASE FOR THE EARLY DIAGNOSIS OF LYSOSOMAL STORAGE DISORDERS

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CONTRIBUTION OF THE MAIN AUTHOR AND CO-AUTHORS

Haya Abdulkarim conceived and designed the analysis, collected the data, contributed data or analysis tools, performed the analysis and wrote the paper.

Prof. Mohamed Siaj conceived and designed the analysis, contributed data or analysis tools, and modification and correction.

3.1 Abstract

Pompe, Gaucher and Krabbe disease are Lysosomal storage disorders (LSDs) which are a group of genetic diseases that causes the accumulation of lipids in tissues and cells. Pompe, Gaucher and Krabbe are characterized by the deficiency of acid α-glucosidase (GAA), β-Glucocerebrosidase (GBA) and galactocerebrosidase (GALC), and treatable if detected in their early stages. Here, we present the fabrication of an electrochemical immunosensor for the multiplexed quantification and simultaneous detection of GAA, GBA and GALC. The sensor was developed by electrodepositing gold nanoparticles (AuNPs) on an array of carbon electrodes, followed by the immobilization of GAA, GBA and GALC specific antibodies via functionalization with cysteamine and glutaraldehyde. The multiplexed immunosensor was able to successfully detect GAA, GBA and GALC at the femtomolar level with respective low detection limits of 0.12 pg/ml, 0.31 pg/ml and 0.18 pg/ml. The immunosensor showed good selectivity, sensitivity and good recovery when spiked in human serum, which confirms its possible applicability in point-of-care testing for the early diagnosis of LSDs.

3.2 Introduction

Lysosomal storage disorders (LSDs) are a group of inherited genetic diseases that causes lipids to accumulate in tissues and cells (Schulze et Sandhoff, 2011), resulting from mutations in genes encoding intralysosomal enzymes (Braunwald *et al.*, 2001; Marsden et Levy, 2010; Wenger *et al.*, 2003). All LSDs share the same characteristic that they cause accumulation of naturally degraded substrates in lysosomes (Meikle *et al.*, 1999), they cause the accumulation of substrates leading to destruction and dysfunction of cells, consecutively, causing tissue dysfunction (Marsden et Levy, 2010; Wilcox, 2004). The severity of the LSDs depends on the nature and quantity of the accumulated substrate (Wilcox, 2004). LSD patients appear normal at birth, but they develop symptoms early in childhood (Meikle *et al.*, 2006). Neurological symptoms include brainstem dysfunction and seizures, and peripheral symptoms include kidney and heart injuries, muscle atrophy, ophthalmic diseases, enlargement of liver and spleen and irregular bone development (Futerman et Van Meer, 2004). However, treatment is available for most LSDs if discovered early in the infantile stage, such as enzyme replacement therapy (ERT), bone marrow or stem cell transplantation and gene therapy, therefore early detection of LSDs is crucial (Meikle *et al.*, 2006; Wilcox, 2004).

Pompe disease (glycogen storage disease type II) is a LSD caused by the deficiency of the lysosomal enzyme acid α-glucosidase (GAA). GAA is necessary in degrading glycogen to glucose thus its deficiency leads to the accumulation of glycogen in organelles (Kishnani *et al.*, 2006; Lim *et al.*, 2015). As treatment for pompe, acid α-glucosidase enzyme is given to patients as ERT, it breaks down glycogen to glucose to reduce its buildup in cells, however, treatment with acid α-glucosidase should be started as early as possible in infants (Kuperus *et al.*, 2017). Another LSD is Krabbe disease (KD) or Globoid cell leukodystrophy (GCL), is a neurological condition caused by the deficiency of galactocerebrosidase (GALC). GALC enzyme is essential for the degradation of galactosylceremide in the white matter of the cerebrospinal nervous system. Krabbe becomes clinically apparent within 6 months of birth and ends in death within 24 months if not treated (Oehlmann *et al.*, 1993; Wenger *et al.*, 1997). Gaucher disease is the most common sphingolipidosis, it results from the deficiency in β-Glucocerebrosidase (GBA) (Sidransky, 2004), it causes the accumulation of glucosylceramide in macrophages (Stirnemann *et al.*, 2017), and it is classified into three main subtypes based on the presence or absence of neurological involvement (Zimran, 2011). Among all LSDs, Pompe, Gaucher and Krabbe diseases are the most common and have severe symptoms that end with mortality. However, these diseases have treatments available, and are detectable in their infantile stage. Hence, if detected and diagnosed early in the patient's infantile stage, treatments can be administered accordingly.

It was proven that the initial identification of LSD patients can be achievable by immunoquantification of lysosomal enzymes and proteins, since mutations lead to not only deficiency in the enzyme activity but also cause diminishment in the amount of protein (Meikle *et al.*, 2006). Deficient patients have protein levels lower than the cut-off concentration which is about 3-100 ng/ml in healthy individuals (Umapathysivam *et al.*, 2000). Previously used methods for the quantification of some LSD related proteins were reported such as fluorescence, tandem mass spectrometry (LC-MS/MS) and immunoassays like enzyme linked immunosorbent assay (ELISA) (Marsden et Levy, 2010; Meikle *et al.*, 2006). Nonetheless, these methods are known to be time consuming and take long analysis time, costly, require specialized laboratories and require large sample volume, making them not ideal for point-of-care testing (POCT)(Want *et al.*, 2005). POCT allows rapid access to results therefore

providing faster monitoring, choice of treatment, prognosis and diagnosis of diseases, resulting in better decision making which is often vital to patient's health. To achieve POCT in the most effective way, methods that are more cost-effective and rapid are being developed (St John, 2010) .

In order to expedite and facilitate clinical diagnosis and POCT, multiple analysis of different biomarkers produce faster and more accurate results. Thus, multiplexed analysis utilizing a single analytical device holds a great promise in upgrading and simplifying diagnostic procedures, as it provides more data, quicker. Multiplexed detection provides numerous advantages such as utilizing less sample volume, less averaged analysis time, more statistically reliable conclusions and more informative detection outcomes (Rosa *et al.*, 2022).

Biosensors are evolving to be an interesting cheaper, simpler and more sensitive alternatives for conventional methods of detection for diagnostic purposes. More specifically, electrochemical immunosensors are being researched extensively in the field of biomedical research and diagnostics, due to their high sensitivity, rapid response, minimization of sample volume used, their capability of being miniaturized and their ability of multiplexing. Electrodepositing electrochemical immunosensors with gold nanoparticles (AuNPs) via chronoamperometry, improves its performance via the enhancement of electron transfer rate and catalytic activity of the sensor (Eissa, Shimaa *et al.*, 2018). It is done by reducing $HAuCl_4$ using potassium nitrate. AuNPs increase the surface area allowing more antibodies to immobilize to the surface of the transducer resulting in a significantly higher signal and sensitivity. Gold nanoparticle-modified electrodes demonstrated a nearly threefold increase in electroactive area, resulting in an increase in functional density of biomolecules as well as improved electron exchange and sensitivity(Elshafey *et al.*, 2016; Elshafey, Reda *et al.*, 2013).

In this work, we report a novel multiplexed electrochemical immunosensor developed for the quantification and simultaneous detection of GAA, GBA and GALC. Carbon microarray disposable chips electrodeposited with AuNPs were utilized due to their high conductivity and high surface area that allows the immobilization of more antibodies. Antibodies for GAA, GBA and GALC were immobilized on the sensor for the detection of the proteins. This multiplexed

sensor could be utilized in the crucial early diagnosis of LSDs in newborns in order to administer the right treatment.

3.3 Experimental

3.3.1 Materials and reagents

Cysteamine hydrochloride, ethanolamine, potassium ferrocyanide ($K_4Fe(CN)_6$) potassium ferricyanide ($K_3Fe(CN)_6$), glutaraldehyde, phosphate buffered saline tablets (PBS 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, at pH 7.4), potassium nitrate, gold chloride solution (HAuCl4), and Human Serum (from male AB clotted whole blood) were obtained from Sigma-Aldrich (Oakville, ON, Canada). ELISA kits for Acid α-glucosidase (GAA), β-glucocerebrosidase (GBA) and Galactocerebrosidase (GALC) were purchased from LSBio (Seattle, WA, US). Proteins and monoclonal antibodies for Acid α-glucosidase (GAA), β-glucocerebrosidase (GBA) and Galactocerebrosidase (GALC) were also acquired from LSBio.

3.3.2 Instrumentation

All electrochemical measurements were done in 5 mM $Fe(CN)_6^{-3/-4}$ redox couple prepared in 10 mM PBS buffer solution (pH 7.4). Electrochemical measurements were performed using AUTOLAB pgSTAT302N (Metrohm, Netherlands) potentiostat, connected to a computer with Nova 1.9 software. Multiplexed immunosensors were fabricated using carbon microarray disposable chips, chips and their corresponding connectors were obtained from BioDevice Technology Ltd. (Nomi, Japan). Each array chip contains eight individual working electrodes, a silver/silver chloride reference electrode and a carbon counter electrode. Parameters for square wave voltammetry SWV measurements are (initial potential 0.4 V, end potential -0.4 V, step potential -0.005 V, amplitude 0.02 V, frequency 25 Hz, interval time 0.04 s and scan rate 0.125 mV/s.

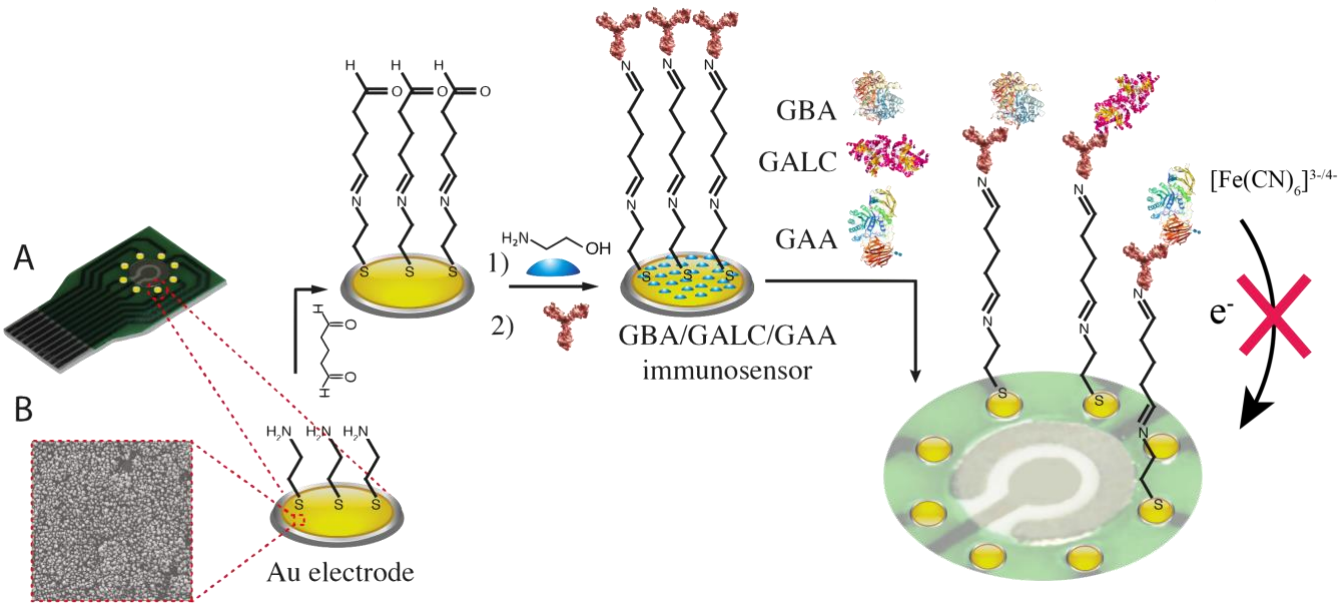
3.3.3 Methods

3.3.3.1 Au electrodeposition and fabrication of the immunosensor

A disposable array chip of eight working electrodes (BioDevice Technology Ltd., Japan) was electrodeposited with AuNPs using chronoamperometry. A solution of 0.1 M KNO_3 containing

6 mM $HAuCl_4$ was placed on the sensor's surface, and it was connected to a potentiostat. Chronoamperometry was applied at a potential of -0.85 V over a period of 200 seconds for each working electrode until the surface turns visibly golden (See Appendix A for supplementary information). Chips were then washed with deionized water and dried then stored for further use.

The multiplexed immunosensor was fabricated on the electrodeposited array chip as indicated in (Scheme 2.1). First, the array chip containing eight electrodes electrodeposited with AuNP was incubated with 10 mM cysteamine hydrochloride solution for 2 hours to form self-assembled monolayers (SAM), then washed with deionized water. Consequently, the modified electrodes were incubated with 2.5% glutaraldehyde solution in phosphate buffered saline (PBS) pH 7.4 for 1.5 hours to activate of the surface, then rinsed with PBS pH 7.4. Electrodes were then incubated with 10 µg/ml of GAA, GBA and GALC antibody solutions prepared in PBS pH 8.5, each solution was added to a different working electrode on the same chip, they were kept for 1.5 hours. After careful washing with PBS pH 7.4 to remove non-bound antibodies, 0.1 M ethanolamine was added and incubated for 30 minutes to block any non-reactive sites. After blocking, the chip was rinsed again with PBS pH 7.4 and stored in a water-saturated container at 4 ˚C. The electrodes were incubated in all steps at room temperature and in a humid environment.



Scheme 3.1 Schematic diagram of the fabrication steps and detection mechanism of the multiplexed immunosensor for GAA, GBA and GALC (A). SEM image of an electrode after electrodeposition of AuNPs via chronoamperometry (B). The AuNPs-modified electrodes were then functionalized with cysteamine to form SAMs, followed by glutaraldehyde as crosslinker. The binding of the analytes to the antibodies hinders the access of the solution-based redox probe to the electrode surface (red Cross).

3.3.3.2 Response and detection of the immunosensor

Each modified electrode was incubated with different concentrations of their specific proteins (0.1 pg/ml to 10 ng/ml for GAA), (0.1 pg/ml to 2 ng/ml for GBA) and (0.1 pg/ml to 2.5 ng/ml for GALC) in PBS pH 7.4. The electrodes were then washed with PBS pH 7.4 and then measured in redox system of $Fe(CN)_6^{-3/-4}$ using SWV. For selectivity experiments, electrodes were incubated with 0.5 ng/ml of their other non-specific proteins, followed by measuring the signal. To calculate the sensor's signal, the percentage of decline in the SWV peaks obtained before and after protein binding to the antibodies was utilized and calculated as ((i°-i)/i° %), where (i) indicates the current after incubation with the proteins, and (i°) is the current measured before incubation at the control concentration of zero.

3.3.3.3 Application of GAA, GBA and GALC immunosensor in human serum

Human serum (from male AB clotted whole blood, Sigma Aldrich, Canada) was diluted 1:100 with PBS pH 7.4, divided and spiked with 10 ng/ml of GAA, and 1 ng/ml of GBA and GALC, each was incubated on the chip for 30 minutes at room temperature and then washed with PBS pH 7.4. SWV was then measured for each spiked sample and recovery % was obtained. Results were compared with commercial ELISA kits for each protein.

3.3.3.4 ELISA for GAA, GBA and GALC in serum

Triplicates of ELISA were performed for GAA, GBA and GALC in accordance with the manufacturer's protocol. Primarily, the standards and spiked diluted serum samples were incubated in the antibody precoated wells at 37 °C for 1 hour (GAA and GBA) and 2 hours (GALC). After washing, 100 µL of Biotin-conjugated secondary antibodies were incubated in the wells for 1 hour at 37 °C. Wells were washed and then incubated with 100 µL of streptavidin-Horseradish Peroxidase (HRP) complex at 37 °C for 1 hour (GALC) and 30 minutes (GAA-GBA). Next, 100 µL of 3,3′,5,5′-Tetramethyl-benzidine (TMB) substrate solution were added to the wells and incubated for 10–20 minutes, then stopped by adding 50 µL of sulfuric acid stop solution. Readings were taken at 450 nm using a microplate reader.

3.4 Results and Discussion

3.4.1 Construction of the immunosensing platform

One-use array carbon electrodes were used to fabricate the multiplexed immunosensor (Scheme 3.1). The first step was to modify the carbon working electrodes by electrodeposition of Au particles by reducing KNO_3 solution of $HAuCl_4$ by chronoamperometry. When the $HAuCl_4$ is reduced, it forms an intense layer of AuNPs on each carbon electrode surface that can be seen clearly. Thiol-gold chemistry was utilized for surface coating of AuNPs, it forms a strong and stable gold-sulfur bond that allows strong anchoring of biological elements. Therefore, AuNPs-modified electrodes were then functionalized with cysteamine to form SAMs, followed by glutaraldehyde which is a homobifunctional crosslinker that binds the amine group from the cysteamine to one of aldehyde groups to form an imine bond, and the other aldehyde group to bind with an amine group from the antibodies. GAA, GBA and GALC antibodies are therefore immobilized on each electrode individually. The immunosensors were

then ready to be used for detection after the electrodes were blocked with ethanolamine to eradicate non-specific binding and to prevent false signals. The stability of the immunosensor was examined by preparing an immunosensor and measuring it's SWV peaks, and then storing it in a humid container at 4 ˚C for 1 month. After a month, SWV peaks were measured again and the response change before and after storing was found to be only (2.2-3%), which indicates the stability of the immunosensor under long-term storage conditions.

3.4.2 Electrochemical characterization of the fabrication steps

SWV was measured for each step of the immunosensor fabrication, in redox couple solution of $Fe(CN)_6^{-3/-4}$ as shown in Figure 3.1. Bare carbon was measured before electrodeposition with AuNPs, it showed a small peak current. After AuNP deposition a significant increase in current was observed which is attributed to the higher surface area of AuNPs and faster electron transfer compared to carbon. After functionalization with cysteamine and the formation of SAM on AuNPs, there was a further increase in the peak current due to the positive charge of amine groups formed. Following the addition of the crosslinker glutaraldehyde and the immobilization of antibodies for GAA, GBA and GALC, peak currents for each sensor decreased differently because of the conformational and size changes between antibodies.

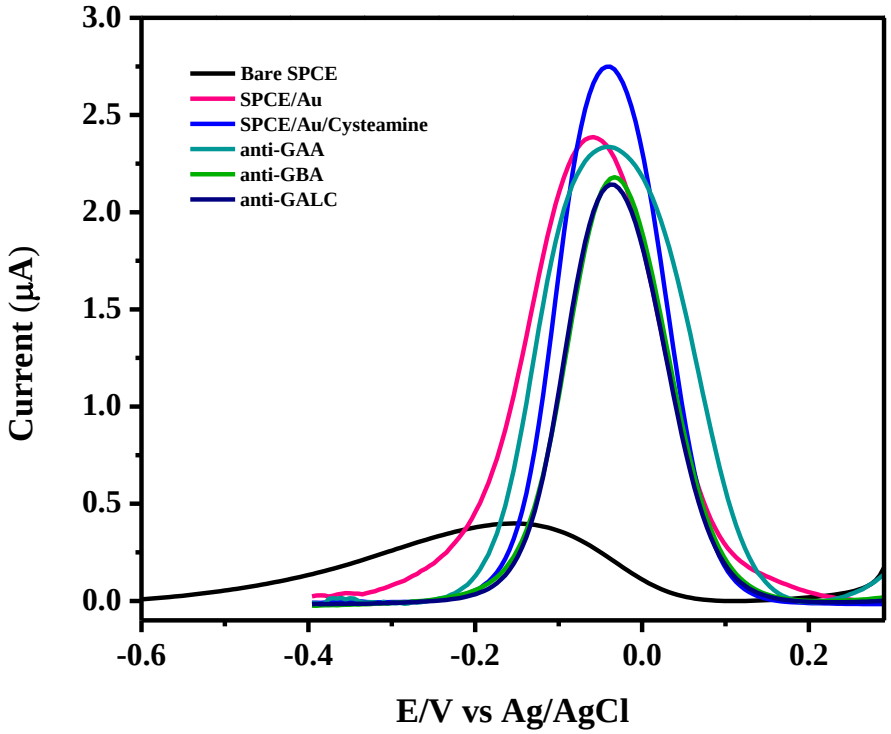


Figure 3.1 SWV peaks for the characterization of the fabrication steps of the immunosensor before, after AuNP electrodeposition and after immobilization of antibodies and blocking with ethanolamine.

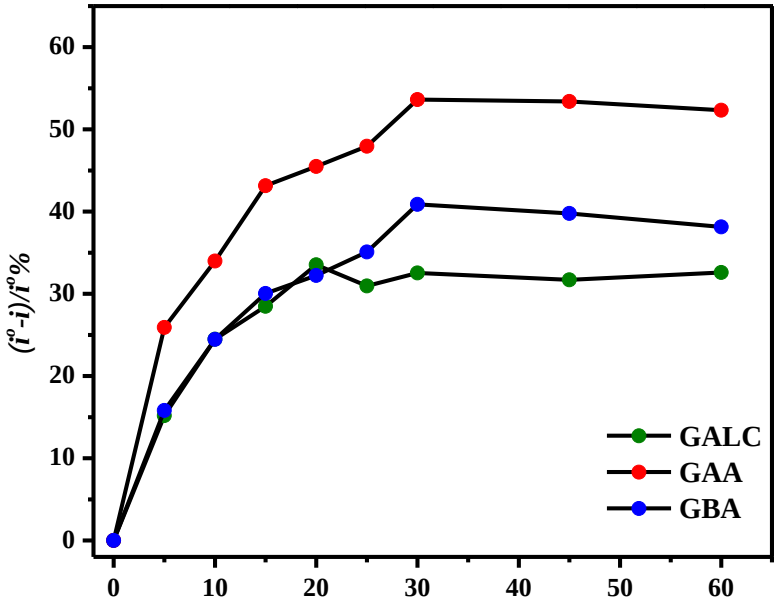


Figure 3.2 Plot of the immunosensor response ((i˚-i)/i˚ %) versus the binding time in minutes of the immunosensors with their specific proteins for GAA, GBA and GALC.

3.4.3 Binding of the immunosensor and the time optimization of immunocomplex formation

To optimize the minimal time required for the formation of the immunocomplex to get the maximum signal, GAA, GBA and GALC multiplexed immunosensor was incubated with protein solutions for different time periods starting with 5 minutes up to 1 hour. As shown in Figure 3.2, after each incubation, SWV was measured in reference to the signal of the immunosensor. The sensor response was calculated using ((i˚-i)/i˚ %) and a continuous increase in antibody-antigen binding was detected after each incubation. This experiment was repeated three times. After 30 minutes of incubation no signal enrichment was observed, consequently, the optimum time to form the immunocomplexes were 30 minutes for GAA and GBA, and 20 minutes for GALC.

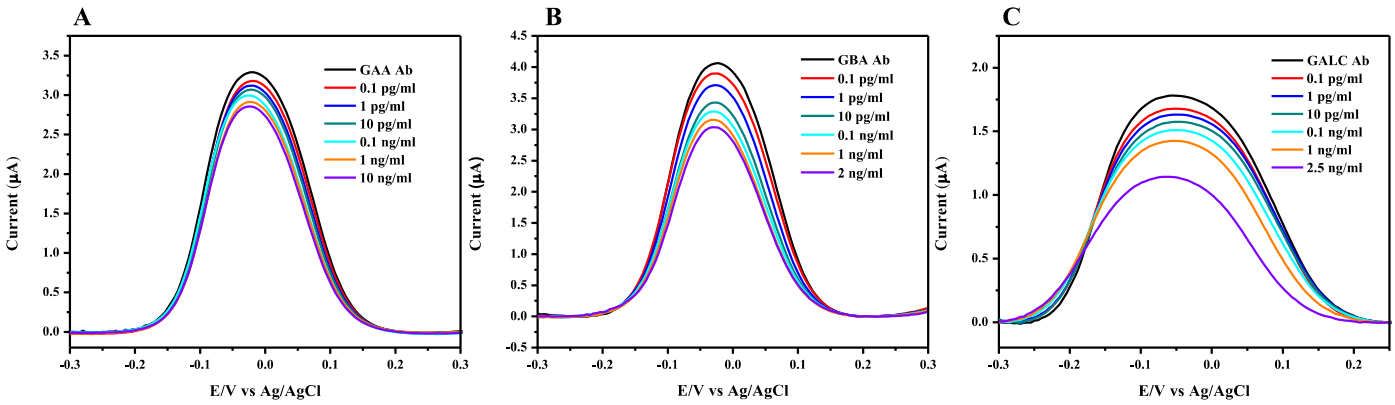


Figure 3.3 SWV peaks measured before and after binding with different concentrations of GAA (A), GBA (B) and GALC (C).

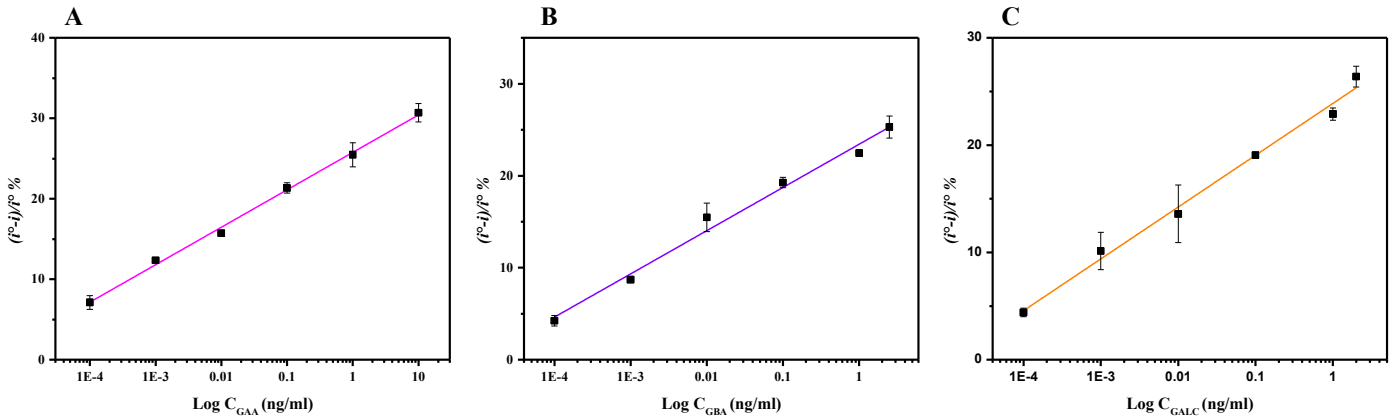


Figure 3.4 Calibration curves plotting the Log of the different concentrations (ng/ml) of GAA (A), GBA (B) and GALC (C) versus the immunosensor's response ((i°-i)/i° %). Error bars show the standard deviation of the measurements.

3.4.4 Immunosensor's detection of GAA, GBA and GALC

The multiplexed immunosensor was incubated in prepared solutions of different concentrations of the proteins GAA, GBA and GALC. SWV was measured after each incubation to determine the binding of proteins. Figure 3.3 shows SWV peaks obtained after binding, as the concentration was increasing, the commensurate peaks were decreasing gradually. Figure 3.4 shows the corresponding calibration curves displaying adequate linearity. GAA showed a linear behavior between 0.1 pg/ml and 10 ng/ml, linear behavior was also observed between 0.1 pg/ml to 2 ng/ml for GBA, and between 0.1 pg/ml to 2.5 ng/ml for GALC. The error bars in calibration curves represent SD of triplicate measurements. For each immunosensor, limit of detection (LOD) was calculated, 0.12 pg/ml (1.5 fM) for GAA, 0.31 pg/ml (5.19 fM) for GBA, and 0.18 pg/ml (2.25 fM) for GALC, indicating the high sensitivity of the immunosensor at the femtomolar level. The LOD values were compared to the detection limits of the commercial ELISA kits available for the proteins, as LODs obtained were 0.9, 12.8 and 78 pg/ml for GAA, GBA and GALC, respectively. The multiplexed immunosensor showed to be superior to ELISA by having a lower LOD, being label-free, faster, cost-effective, ability to simultaneously detect multiple analytes in one run and their promising applicability in point-of-care testing.

3.4.5 Selectivity of the multiplexed immunosensor

The multiplexed immunosensor's selectivity was tested by incubating the electrodes in 0.5 ng/ml of GAA, GBA and GALC individually. Each immunosensor response was measured before and after incubation with the non-specific proteins, then washed with PBS pH 7.4. Figure 3.5 shows that each immunosensor had the highest signal and affinity to their corresponding protein in comparison to the non-significant signal obtained after incubation with the other non-specific proteins. This confirms the high selectivity and specificity and low cross-reactivity of the multiplexed immunosensor against GAA, GBA and GALC. The simultaneous detection was validated by preparing a mixture containing 0.5 ng/ml of GAA and GBA and incubating it on each of the sensors on the chip. Specific significant signals were obtained only for GAA and GBA compared to GALC as shown in Figure 3.6, confirming its ability of simultaneous detection.

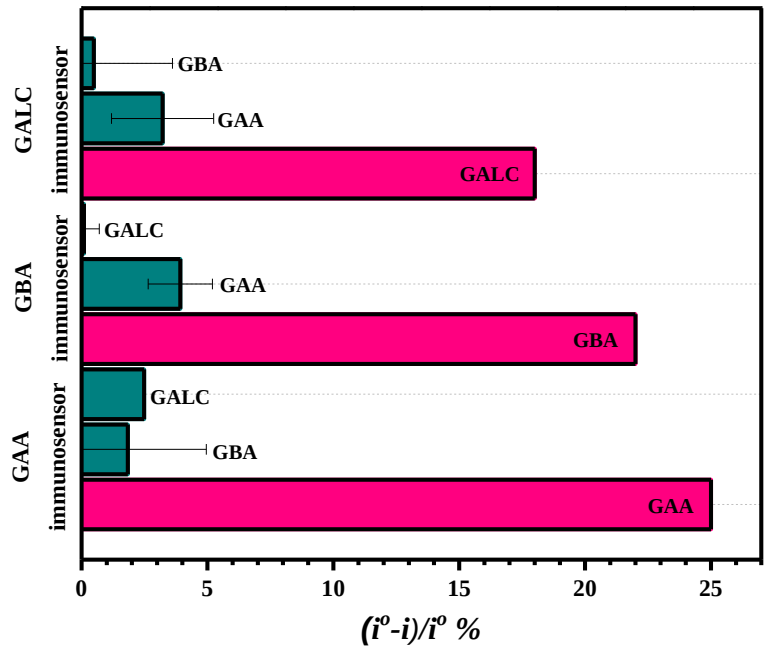


Figure 3.5 Immunosensor's responses to 0.5 ng/ml to GAA, GBA, GALC and BSA. Error bars represent SD.

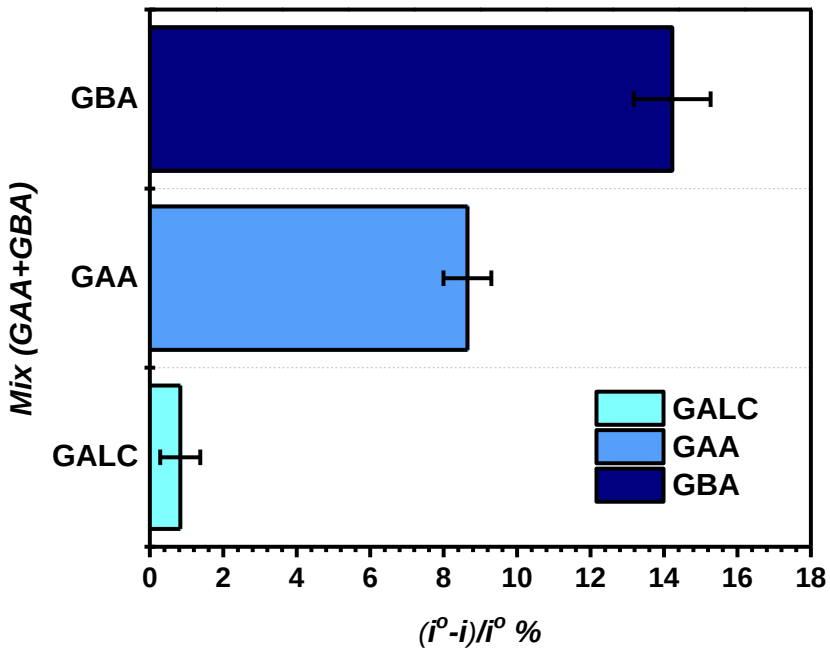


Figure 3.6 GAA, GBA and GALC sensors' response after incubation each with a mixture of 0.5 ng/ml of GAA and GBA.

3.4.6 Application of the immunosensor in spiked serum

To assess whether the immunosensor would be applicable to test biological samples obtained from patients for rapid and sensitive quantification of GAA, GBA and GALC for the initial diagnosis of LSDs, immunosensors were tested on spiked samples of human serum. Spiked serum samples were also analyzed with commercial ELISA kits (LSBio) specific for GAA, GBA, and GALC for comparison. After measuring each serum sample, recovery was studied by spiking serum samples with GAA, GBA and GALC, the recovery results are shown in **Table 3.1**. The good recovery percentages suggest the immunosensor's high precision and eradicates the significant matrix effect, therefore it's applicability in different biological specimens.

Table 3.1 Three human serum samples individually diluted 1:100, spiked with 10 ng/ml of GAA and 1 ng/ml of GBA and GALC, and the calculated recovery % and relative standard deviation (RSD %).

	Spiked concentration *(ng/ml)*	**Measured concentration *(ng/ml)***	**Recovery %**	**RSD %**
GAA	10	10.52	105.2 %	2 %
GBA	1	0.905	90.5 %	10.6 %
GALC	1	0.96	96 %	1.6 %

3.5 Conclusion

In conclusion, a novel multiplexed label-free electrochemical immunosensor was successfully developed for the quantification and simultaneous detection of acid α-glucosidase (GAA), β-Glucocerebrosidase (GBA) and galactocerebrosidase (GALC) for the early detection of LSD diseases; pompe, krabbe and gaucher. This rapid multiplexed sensor was fabricated on AuNPs-modified carbon printed array electrodes. It showed higher sensitivity and lower LOD than commercial ELISA kits. The immunosensor showed very good specificity for each analyte against the other, and a very low LOD of 0.12 pg/ml (1.5 fM) for GAA, 0.31 pg/ml (5.19 fM) for GBA, and 0.18 pg/ml (2.25 fM) for GALC, indicating the high sensitivity of the immunosensor at the femtomolar level, respectively. Very good recovery percentages when measured in spiked human serum, which confirms its possible applicability in POCT for the early diagnosis of LSDs.

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CHAPTER 4

SCIENTIFIC ARTICLE

ULTRASENSITIVE DISPOSABLE LABEL-FREE ELECTROCHEMICAL IMMUNOSENSOR FOR THE MULTIPLEXED DETECTION OF FOUR SEROTYPES OF DENGUE VIRUS

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CONTRIBUTION OF THE MAIN AUTHOR AND CO-AUTHORS

Haya Abdulkarim, Prof. Mohamed Siaj and Prof. Mohammed Zourob conceived the idea of this work.

Haya Abdulkarim designed and performed all the experiments, analyzed the data and wrote the full manuscript.

In collaboration with Prof. Mohammed Zourob from Alfaisal University, all experiments were performed in Prof. Mohammed Zourob's Laboratory at Alfaisal University, Riyadh, Saudi Arabia.

All authors have read, given feedback and approved the current version of the manuscript for publication.

4.1 Abstract

Dengue fever is caused by four identified serotypes of Dengue virus. Dengue NS1 antigen is currently used as a significant diagnostic biomarker and is found in the serum of patients in the early stages of DENV infection. If not detected in the early stages it could develop into severe dengue, therefore its early detection is vital. Herein, we present the development of a label-free electrochemical immunosensor for the simultaneous and multiplexed quantification of Dengue's four serotypes DENV-1, DENV-2, DENV-3 and DENV-4. The sensor was prepared via the immobilization of dengue-specific antibodies onto cysteamine-glutaraldehyde functionalized carbon array chips that were electrodeposited with gold nanoparticles (AuNPs). Square wave voltammetry (SWV) was performed to measure the response as a function of DENV protein concentrations. The findings showed that the sensor was successful in detecting DENV (1-4) in human serum and demonstrated a detection range from 0.1 pg/ml to 100 ng/ml with a very low detection limit. This DENV immunosensor holds great promise as a point-of-care device for the early-stage diagnosis of dengue virus.

4.2 Introduction

Early diagnosis of Dengue fever is critical as it could lead to lethal illnesses known as dengue shock syndrome (DSS) and dengue hemorrhagic fever (DHF) (Omar *et al.*, 2018). Dengue fever is caused by Dengue Virus of the Flavivirus family and is transmitted to humans by Aedes mosquitoes (Martina *et al.*, 2009). There are four serotypes of dengue virus (DENV-1, DENV-2, DENV-3, and DENV-4). Each serotype is comprised of 3 structural proteins (capsid, membrane, envelope (E) protein) and 7 non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) (Pothapregada *et al.*, 2016).

Dengue epidemics inflict issues and high costs on health services and the economic organizations of affected countries (Guzman *et al.*, 2016). An estimated 50 million infections occur each year and more than 2.5 billion people are at risk of infection. Dengue symptoms range from light aches and pains, nausea and rash, to more serious symptoms that are present with warning signs and require strict observation and medical intervention like abdominal pain, persistent vomiting, fluid accumulation, mucosal bleeding and liver enlargement. Patients

exhibiting those symptoms may evolve to severe dengue which is characterized by organ damage (Allonso *et al.*, 2014).

According to current studies, one of the well-known early biomarkers for DENV virus is non-structural protein NS1, released into the blood stream in moderately high concentrations during replication (Dutta *et al.*, 2020). Dengue NS1 antigen, a highly conserved 43-48 kDa glycoprotein, produced in both membrane-associated and secretion forms, is abundant in the serum of patients during the early stages of DENV infection (Wang et Sekaran, 2010; Young *et al.*, 2000). The detection of the viral antigen nonstructural protein 1 (NS1) is used for the early diagnosis of dengue infections (Santos *et al.*, 2020). It is currently used as a significant diagnostic biomarker of infection from the first day after the onset of infection, whereas IgM is generally detectable after 4–6 days. The median NS1 concentration varied from 22.6 ng/mL on day 2 to 36.8 ng/mL on day 7 of infection (Allonso *et al.*, 2014).

Previously, dengue diagnosis was performed via the detection of specific antibodies against DENV (IgM or IgG). Also, detection of viral RNA through reverse transcriptase-polymerase chain reaction (RT-PCR) is one of the currently available laboratory methods for dengue diagnosis. But after they discovered that NS1 circulates in the infected patients' serum, an important progress in early dengue detection method occurred, were several NS1 detection kits were developed. Therefore, it is important to improve current methodologies to detect all serotypes of dengue at an early stage with high sensitivity (Allonso *et al.*, 2014).

Most biosensors used for dengue virus detection use any one of the following three transducers: optical, piezoelectric, and electrochemical transducers. Among all types of biosensors, electrochemical biosensors have gained extreme attention not only in research but also in the commercial sectors. An electrical signal that is generated by the specific interaction/binding to the analyte in a way proportional to the analyte's concentration is measured by electrochemical biosensors. They are of great interest due to their ability to be miniaturized, relatively low cost, high stability, specificity, and selectivity. These are preferred over the optical- and mechanical-based sensors because of their remarkable detectability, experimental simplicity, and low-cost fabrication. Immunosensors are simpler, more specific, and convenient than other conventional immunoassay methods. They are divided into two categories: label-free and labeled

immunosensors. Label-free immunosensors are designed such that the immunocomplex (i.e., the antigen–antibody complex) is directly determined by measuring the physical changes induced by the formation of other complex. In contrast, in labeled immunosensor, a sensitively detectable label is incorporated and the immunocomplex is thus sensitively determined through measurement of the label (Dhal *et al.*, 2020). Label-free electrochemical biosensors deliver better performances in terms of the detection limits and ranges, where the detection limits have reported up to the sub-nanomolar level (sometimes in picomolar level) with wider detectable linear ranges. Therefore, various researchers established new electrochemical immunosensing platforms for the quantification of dengue viruses.

Modifying the transducer part of the biosensor with nanomaterials like nanoparticles creates a rougher surface. Combining gold nanoparticles (AuNPs) with signal amplifying technologies is a common way to increase the detection sensitivity of electrochemical biosensors (Jiang *et al.*, 2018), by providing higher surface area for immobilization of additional detecting biomolecules. Some of the advantages of this novel developed immunosensor is its low cost, high efficiency, rapid response, and high sensitivity compared to other devices of lower sensitivity and higher cost with possible false-positive results, as well as the low detection limits. In this work we present the construction and detection performance of a novel label-free immunosensor for the simultaneous quantification of four different serotypes of Dengue virus NS1 antigen. Therefore, this proposed immunosensor could be applied as a point-of-care device for a precise diagnosis of DENV infection in its initial stage, presenting excellent results for the serum sample detection methodology.

4.3 Experimental

4.3.1 Reagents and materials

Dengue Virus NS1 Protein Serotypes 1-4 (containing 1 vial of DENV1-NS1 from strain Nauru/Western Pacific/1974, 1 vial of DENV2-NS1 from strain Thailand/16681/84, 1 vial of DENV3-NS1 from strain Sri Lanka D3/H/IMTSSA-SRI/2000/1266, and 1 vial of DENV4-NS1 from strain Dominica/814669/1981) and their antibodies (Mouse Anti-Dengue Virus Pan-Serotype NS1 Antibody (DA034)). cysteamine hydrochloride, ethanolamine, potassium ferrocyanide ($K_4Fe(CN)_6$) potassium ferricyanide ($K_3Fe(CN)_6$), glutaraldehyde, phosphate

buffered saline tablets (PBS 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, at pH 7.4), potassium nitrate, gold chloride solution ($HAuCl_4$), and Human Serum (from male AB clotted whole blood) were obtained from Sigma-Aldrich (Oakville, ON, Canada).

4.3.2 Instrumentation

All of electrochemical measurements were done using AUTOLAB potentiostat model pgSTAT302N (Metrohm, Netherlands), coupled to a computer with FRA32M module and Nova 1.11 software. Microarray chips of carbon and their corresponding connectors were obtained from BioDevice Technology Ltd. (Nomi, Japan). Each chip consists of carbon working electrodes, carbon auxiliary electrode and Ag/AgCl reference electrode. Electrochemical techniques were performed in an aqueous solution of $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ 5.0 mM containing PBS pH 7.4. SWV measurements were done in the potential range of 0.4 V to -0.6 V, step potential − 0.005 V, amplitude 0.02 V, frequency 25 Hz, interval time 0.04 s and scan rate 0.125 mV/s.

4.3.3 Methods

4.3.3.1 Fabrication of the biosensing platform

Electrodeposition of gold nanoparticles (AuNPs) was done first on the working electrodes of printed multiplexed array chips. Electrodeposition was done by first preparing a 6 mM solution of $HAuCl_4$ in 0.1 M KNO_3 and then placing it on the surface of the electrode. The electrode was then connected to the potentiostat and the electrochemical technique used was chronoamperometry at potential -0.95 V for 200 s until the working electrodes turned visibly golden and was then dried.

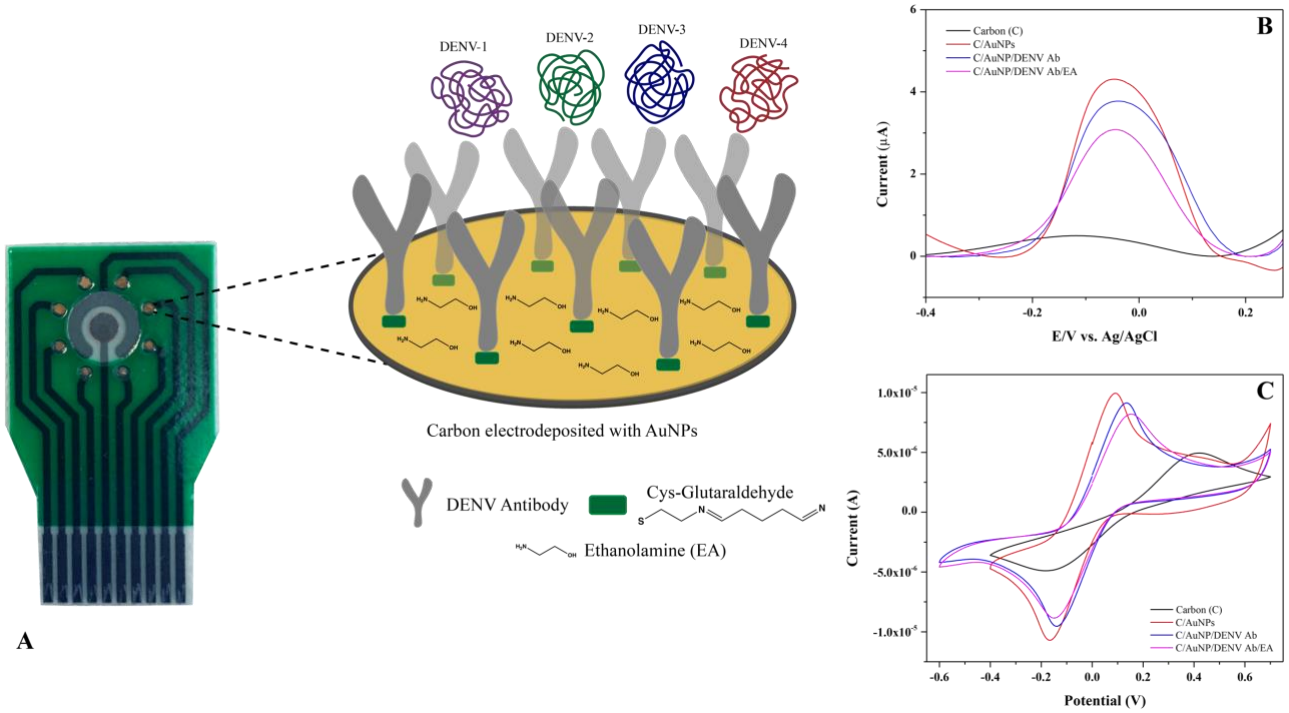

Figure 4.1 Diagram showing the components of the fabricated immunosensor on carbon electrodes that were electrodeposited with AuNPs via chronoamperometry (A). SWV (B) and CV (C) peaks obtained/measured for the electrode before, after AuNPs electrodeposition, after AuNPs electrodeposition, after immobilization of DENV antibodies and after blocking.

Figure 4.1 A shows the construction process of the multiplexed sensor. Following the electrodeposition of AuNPs on the chip's working electrodes' surface, they were modified with cysteamine hydrochloride 10 mM solution in water and left at room temperature for 2 h. To activate the surface after the formation of self-assembled monolayers, electrodes were washed with water and then incubated in 2.5% glutaraldehyde solution prepared in PBS pH 7.4 for 1.5 h then rinsed again with PBS pH 7.4. A diluted solution of 10 ug/ml DENV antibodies in PBS pH 8.5 was made and placed on the electrodes for at least 1.5 h. Working electrodes were then

washed with PBS pH 7.4 to remove non-bound antibodies and then consequently incubated with 0.1 M ethanolamine solution for 30 min to block any remaining activated sites. They were washed again with PBS pH 7.4 and either stored in a humid and cold environment of 4 °C or immediately used for further experiments.

4.3.3.2 Detection and response of the multiplexed sensor

Serial dilution of each of the serotypes was prepared in PBS pH 7.4 to constitute different concentrations of the analytes (0.0001, 0.001, 0.01, 0.1, 1, 10, 100 ng/ml). Blank sample of buffer only was measured first to indicate the signal at zero concentration of each of the analytes. Then each concentration was incubated on the electrode and SWV was measured after each concentration. For verifying the selectivity of this immunosensor, electrodes were incubated with 1 ng/ml of Rift Valley virus and antigens of other viruses from the same family as Dengue (Flavivirus) such as Zika, West Nile and Yellow Fever.

Determining the sensor's signal was done by obtaining the SWV peaks before binding of the antibodies to any analyte, and after incubation with each of the analyte concentrations starting with 0.0001 ng/m until 100 ng/ml. The diminution percentage in SWV peaks obtained when measuring each concentration was calculated as ((i°- i)/i° %), where i° is the measurement of current of analyte at concentration zero, and i is the current obtained after incubation with each of the concentrations of analyte.

4.3.3.3 Sensor's performance in human serum samples

Spiked serum samples were prepared by diluting human serum (from male AB clotted whole blood, Sigma Aldrich, Canada) 1:100 with PBS pH 7.4, divided into 4 tubes and each was spiked with 1 ng/ml of DENV serotypes. Samples were incubated on the sensor's surface and SWV was measured after.

4.4 Results and Discussion

4.4.1 Development of the multiplexed immunosensor

Disposable array chips were used, and the first step was to modify their working electrode surface with AuNPs, via the reduction of $HAuCl_4$ by applying chronoamperometry. This

caused the formation of a rich layer of gold nanoparticles on the surface. The modification of AuNPs with cysteamine introduces amine groups after the adsorption on the gold's surface via thiol groups forming self-assembled monolayers (SAMs). The glutaraldehyde added will covalently bind with the amine groups, and then the glutaraldehyde will react with the DENV antibodies to give a specific linkage to capture DENV antigens and guarantee specific antigen-antibody binding. To block unreacted active groups and non-specific binding on the sensor's surface, ethanolamine was added to the surface of the electrodes.

4.4.2 Electrochemical characterization

After each step of the construction of the sensor, SWV and CV were measured in $Fe(CN)_6^{3-/4-}$ redox solution. As shown in figure 4.1. B and C, bare carbon was measured prior to electrodeposition of AuNPs, the square wave voltammogram showed small peak current, and a low broad peak with high peak-to-peak separation (ΔE) in the cyclic voltammogram, due to the low electron transfer. After modification of the carbon with gold, both CV and SWV peaks showed a substantial increase in current due to the higher conductivity and faster electron transfer in the redox system, as well as the higher surface area of the nanoparticles. After the functionalization with cysteamine and formation of the SAMs, there was a further increase in the SWV peak current and a minor decrease in the ΔE indicating the enhancement of the electron transfer because of the positive charge of the amine groups that formed. After the addition of glutaraldehyde and the immobilization of antibodies specific for dengue antigen, the peak current decreased further and ΔE further increased as the electron transfer decreased due to the blockage caused by the antibodies.

4.4.3 Optimization of Immunoreaction time

To know the minimal time required for the immunoreaction between the antibody and antigen to happen and give the maximum signal, the immunosensor was incubated with 4 different antigen solutions, each for various time periods starting with 5 min up to 1 h, as shown in figure 4.2 A, B, C and D. SWV was measured after each incubation. There was a gradual increase in signal until 45 min, after that no enhancement was observed. Therefore, it was determined that the optimum incubation time for the sensor in the protein solutions was 45 min for all dengue serotypes.

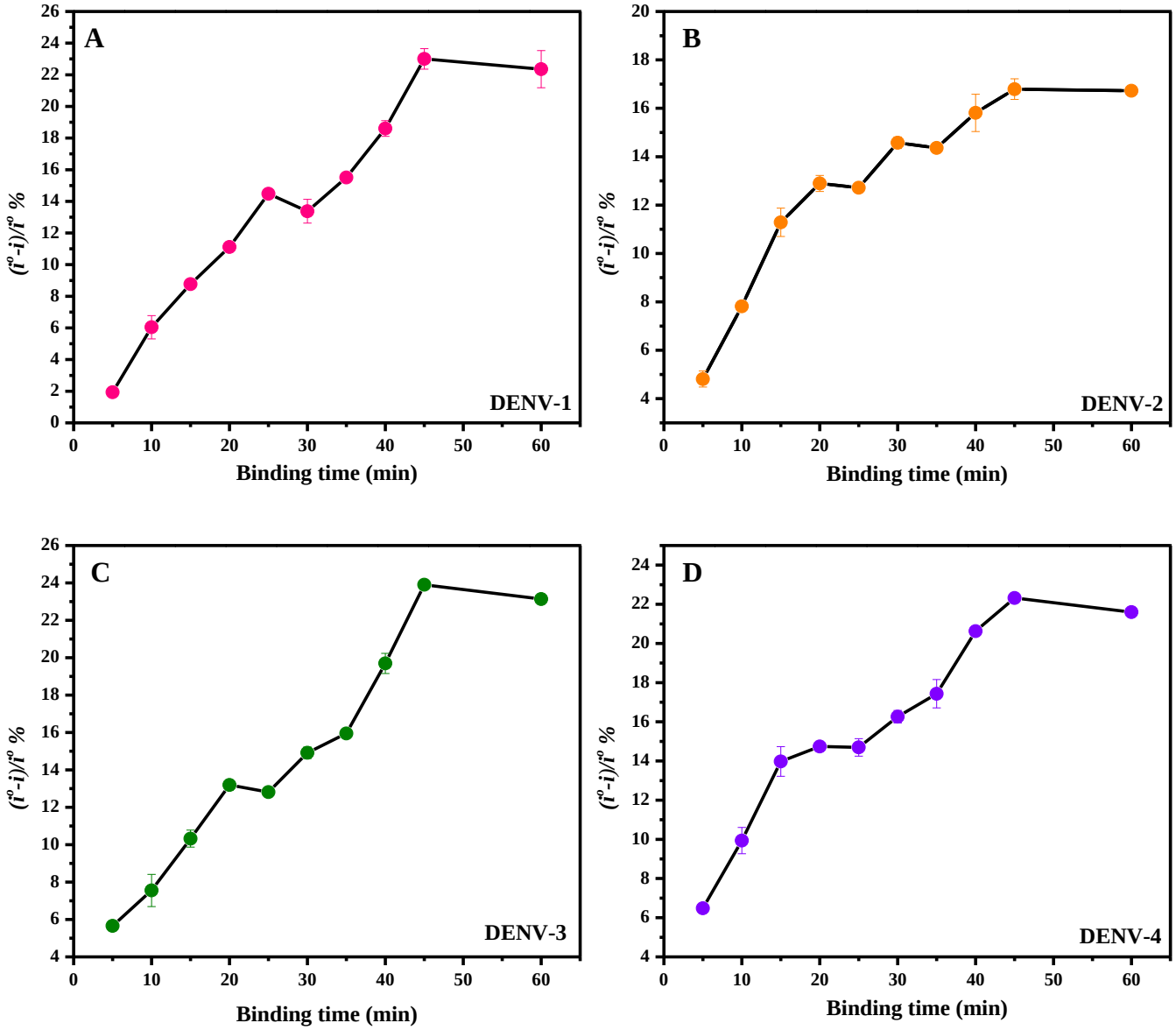

Figure 4.2 Immunosensor's response versus immunocomplex formation time (minutes) of DENV antibodies and their analytes DENV-1 (A), DENV-2 (B), DENV-3 (C) and DENV-4 (D).

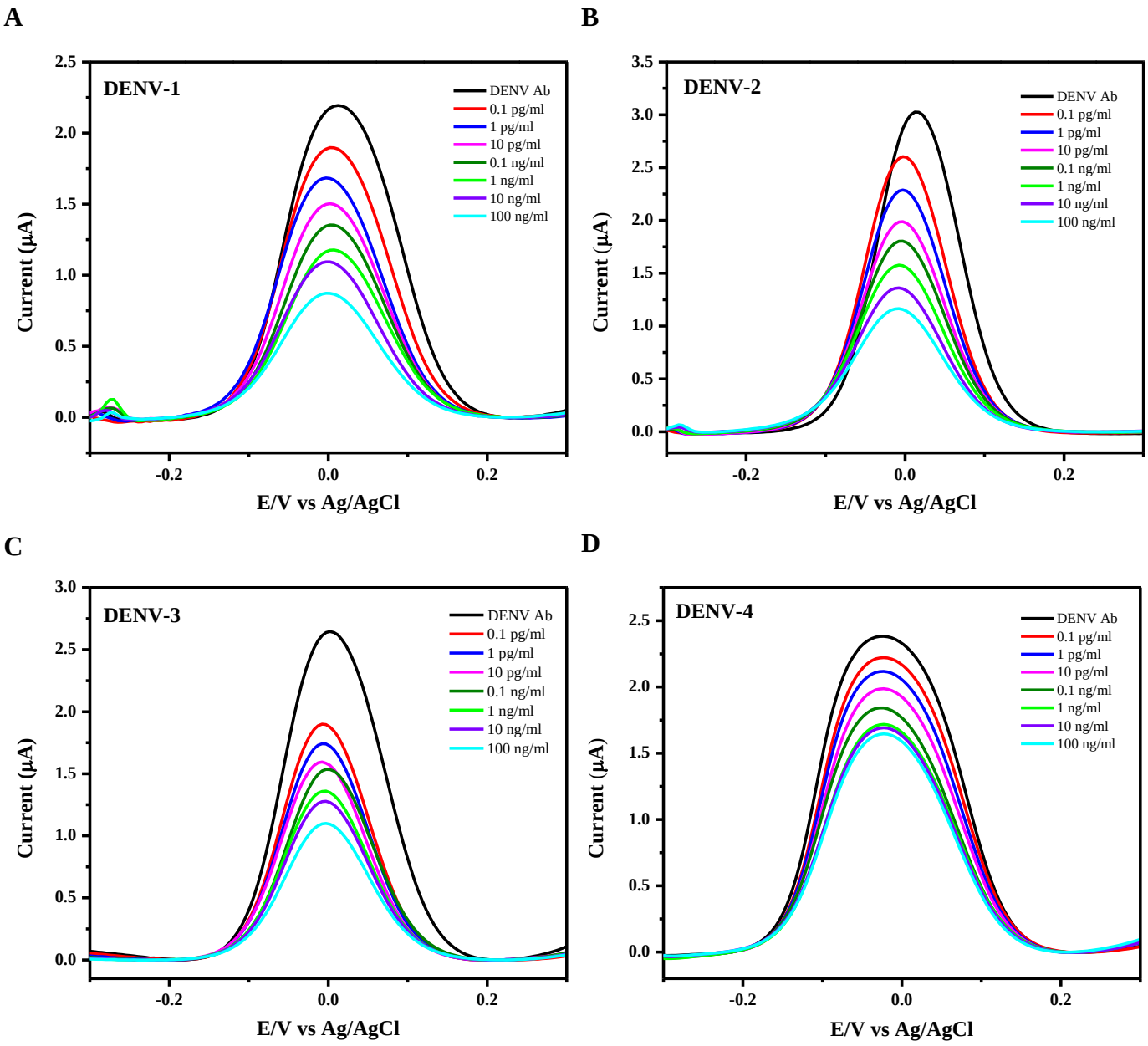

Figure 4.3. SWV peaks measured before and after binding with different concentrations of DENV-1 (A), DENV-2 (B), DENV-3 (C) and DENV-4 (D).

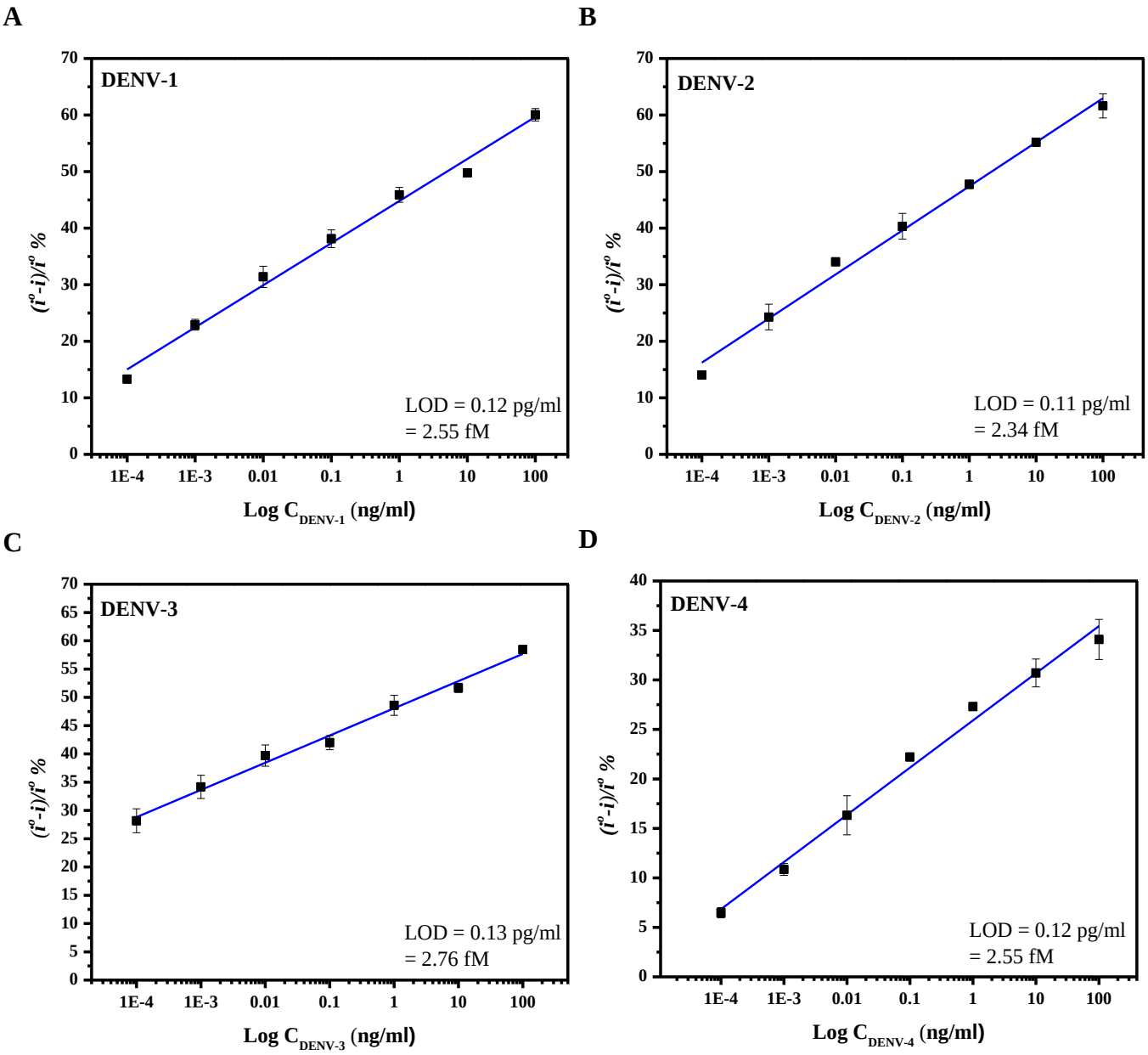

Figure 4.4 Calculated corresponding calibration curves showing the immunosensor's response ((i°-i)/i° %) versus the Log of the different concentrations (0.0001 ng/ml to 100 ng/ml) of DENV-1 (A), DENV-2 (B), DENV-3 (C) and DENV-4 (D).

Table 4.1 Review of previously developed NS1 biosensors for Dengue Virus detection.

Biosensing Platform	Type of Biosensor	Analyte	Linear Range	Limit of Detection (LOD)	Reference
Indium Tin Oxide (ITO)	Impedimetric	NS1	5-4000 ng/ml	35 ng/ml	(Darwish *et al.*, 2015)
BSA-Modified SPCE	Impedimetric	NS1	1-200 ng/ml	0.3 ng/ml	(Nawaz *et al.*, 2018)
SPGE	CV	NS1	1-25 ng/ml	0.5 ng/ml	(Sinawang *et al.*, 2016)
Polyalanine and GC	CV	NS1	1-100 ng/ml	0.1 ng/ml	(Arshad *et al.*, 2020)
AuNPs modified SPEs	Voltametric	NS1 (Serotypes 1-4)	0.1 pg/ml -100 ng/ml	0.12 pg/ml (DENV-1) 0.11 pg/ml (DENV-2) 0.13 pg/ml (DENV-3) 0.12 pg/ml (DENV-4)	This work

4.4.4 Immunosensor's detection of dengue serotypes

Different concentrations of four serotypes of dengue were prepared from (0.1 pg/ml to 100 ng/ml) and incubated on the sensor's surface and then SWV was measured after each incubation. As shown in figures 4.3. A, B, C and D, the SWV peaks gradually decrease as the concentrations of the analyte increases. In the calculated corresponding calibration curves shown in figures 4.4. A, B, C and D, they show very good linearity. Error bars in the curves show the SD of triplicate measurements. The limit of detection (LOD) calculated for DENV-1 was 0.12 pg/ml (2.55 fM), 0.11 pg/ml (2.34 fM) for DENV-2, 0.13 pg/ml (2.76 fM) for DENV-3 and 0.12 pg/ml (2.55 fM) for DENV-4. These LOD represent a very high sensitivity of all serotypes of dengue. Table 4.1. shows a comparison of the biosensing performance and LOD of this fabricated Dengue (NS1) immunosensor with previously reported Dengue biosensors in relevant studies.

4.4.5 Selectivity of the multiplexed immunosensor

The selectivity of the multiplexed immunosensor was tested by incubating it with 1 ng/ml of Rift Valley, Zika, West Nile and Yellow fever. Each sensor's response was measured before and after incubation with the non-specific virus and signal change was calculated. Figure 4.5 shows the drastic difference in the sensor's response to the dengue serotypes and the other viruses. The sensor had very good binding percentage while almost no binding was achieved for the non-specific viruses like Zika. Those results confirm the multiplexed sensor's good selectivity and specificity and low cross-reactivity, as well as the ability of the sensor to simultaneously detect any dengue serotype.

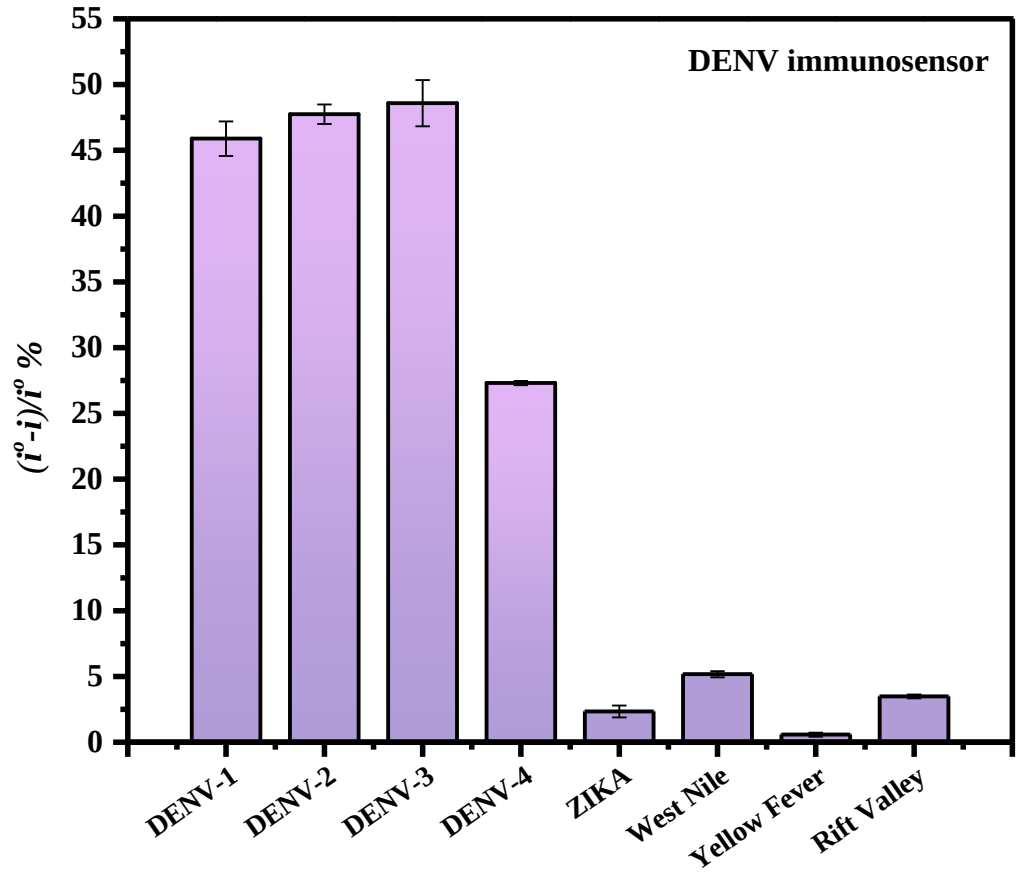

Figure 4.5 Immunosensor's response to 1 ng/ml of DENV (1-4) and to Rift Valley and other viruses.

4.4.6 Applicability of the sensor in human serum

The immunosensor's applicability in real serum samples obtained from patients infected with Dengue virus of any serotype was tested on spiked human serum samples. After measuring each serum sample that had been spiked with dengue virus serotypes 1, 2, 3, and 4, recovery percentages were investigated as shown in Table 4.2. The satisfactory recoveries of 93-97 % suggest the high accuracy of immunosensor and minimizes the matrix effect, therefore suggesting its propitious application in clinical analysis for other biological samples.

Table 4.2 Human serum samples (diluted 1:100) spiked 1 ng/ml of DENV (1-4), recovery %, and the relative standard deviation of three measurements.

	Spiked Concentration (ng/ml)	Measured Concentration (ng/ml)	Recovery %	RSD %
DENV-1	1	0.95	95	3.52
DENV-2	1	0.97	97	2.8
DENV-3	1	0.96	96	1.86
DENV-4	1	0.93	93	1.7

4.5 Conclusion

Aiming to diagnose Dengue virus at its early stage, we developed a label-free electrochemical immunosensor for the simultaneous and multiplexed detection of four serotypes of Dengue. The sensor was developed on carbon array electrodes that were electrodeposited with gold nanoparticles via chronoamperometry. The developed immunosensor exhibited good selectivity for dengue against other viruses like Zika and Yellow fever virus. The sensor showed a very good LOD of 0.12 pg/ml for DENV-1, 0.11 pg/ml for DENV-2, 0.13 pg/ml for DENV-3 and 0.12 pg/ml for DENV-4 demonstrating its high sensitivity. Thus, the developed sensing platform could be implemented as point-of-care testing (POCT) for facilitating early Dengue virus detection.

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CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

The urgency of timely medical diagnosis and early detection of diseases has led to the increased interest and more research focus in this field. Developing novel methods to facilitate early detection of disease biomarkers that are sensitive, fast and cost effective has become an emerging field of research, especially point-of-care testing (POCT) devices as they offer high sensitivity, minimal invasiveness and are cost effective. There are major advances lately in developing sensing platforms like biosensors and more specifically electrochemical immunosensors for the purpose of early detection in the biomedical field.

The main goal of this thesis was to investigate the creation of different label-free ultra-sensitive electrochemical immunosensors, some enhanced with electrodeposited gold nanoparticles (AuNPs) for increased surface area and therefore sensitivity, for detecting biomarkers of cancer drugs, rare disorders, and potential pandemics. Ultimately, the objectives of this work were achieved and the new immunosensors created demonstrated advantages in terms of sensitivity, selectivity, low cost and specificity in detection.

First, to prevent the toxic effects of Lenalidomide, a chemotherapy drug used in the treatment of multiple myeloma (MM), and monitor its efficacy, a new label-free electrochemical immunosensor was developed to quickly detect and measure Ikaros family zinc finger proteins 1 and 3 (IKZF1 and IKZF3). High levels of these proteins in MM patients makes the treatment less effective and can have toxic effects. Therefore, identifying IKZF1 and IKZF3 is important in the treatment of MM. The immunosensors were fabricated by attaching IKZF1 and IKZF3 specific antibodies to gold electrodes using cysteamine and PDITC crosslinkers. These immunosensors were able to detect IKZF1 and IKZF3 proteins with very low detection limits of 0.68 and 0.97 pg/ml respectively. Additionally, they were tested on human serum and were found to be highly selective and sensitive, making them suitable to be used as a point-of-care device in other biofluids, to monitor multiple myeloma patients taking Lenalidomide, to prevent its toxic effects and minimize side effects.

Second, we successfully fabricated a label-free multiplexed electrochemical immunosensor that can detect and quantify GAA, GBA and GALC simultaneously to early diagnose rare diseases Pompe, Gaucher and Krabbe disease. Pompe, Gaucher and Krabbe are Lysosomal storage disorders (LSDs) which are a type of genetic disease that leads to the build-up of lipids in cells and tissues. These disorders are caused by deficiency of acid alpha-glucosidase (GAA), beta-Glucocerebrosidase (GBA) and galactocerebrosidase (GALC) and can be treated if detected early. The sensor was created by adding gold nanoparticles (AuNPs) on carbon electrodes and then attaching GAA, GBA and GALC specific antibodies using cysteamine and glutaraldehyde. The multiplexed immunosensor was able to detect GAA, GBA and GALC at the femtomolar level with low detection limits of 0.12 pg/ml, 0.31 pg/ml and 0.18 pg/ml respectively. It demonstrated good selectivity, sensitivity, and recovery when tested in human serum, confirming its suitability for point-of-care testing for early diagnosis of LSDs.

Third, we developed a multiplexed array platform of label-free electrochemical immunosensor to detect the four serotypes of Dengue virus at an early stage. The sensor was made on carbon array electrodes with gold nanoparticles deposited via chronoamperometry. The sensor showed good selectivity for Dengue virus over other viruses such as Zika and Yellow fever. It also showed a very low detection limit (LOD) of 0.12 pg/ml for DENV-1, 0.11 pg/ml for DENV-2, 0.13 pg/ml for DENV-3 and 0.12 pg/ml for DENV-4, indicating its high sensitivity. This sensor can be used as a POCT for easy detection of Dengue virus.

Finally, detecting diseases early by developing new diagnostic methods can enable earlier intervention and treatment, which can lead to better outcomes for affected individuals. These novel biosensors can provide more accurate and reliable results, which can lead to more effective treatment.

If applied on patients in the future, it could improve the overall public health by enabling early diagnosis and treatment of diseases, reducing healthcare costs, improving the quality of life for affected individuals, identifying rare disorders, detecting asymptomatic cases, simultaneous detection, and enabling point-of-care testing. Biosensors could be used and possibly applied as

point-of-care testing (POCT) which can be used in remote or low-resource settings, making healthcare more accessible. The multiplexed nature of some of the fabricated electrochemical immunosensors allows for simultaneous detection of multiple diseases and biomarkers, which can save time and resources.

Electrochemical immunosensors can be improved in the future and integrated into automated systems, which can improve their efficiency, accuracy, and reduce human error. They can also be incorporated with other technologies, such as microfluidics, lab-on-a-chip, or CRISPR-based systems, which can improve their performance and expand their capabilities. Such biosensors have a lot of potential for future applications in biomedical diagnostics, and research and development in this field continues to improve their sensitivity, specificity, multiplexing capabilities, sample volume, automation, integration with other technologies, and biomarkers discovery.

APPENDIX A

SUPPLEMENTARY INFORMATION FOR:

ULTRASENSITIVE DISPOSABLE LABEL-FREE ELECTROCHEMICAL IMMUNOSENSOR FOR THE MULTIPLEXED DETECTION OF FOUR SEROTYPES OF DENGUE VIRUS

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A.1 Functionalization of array chip with AuNPs

A disposable array chip of eight working electrodes (BioDevice Technology Ltd., Japan) was electrodeposited with AuNPs using chronoamperometry. A solution of 0.1 M KNO_3 containing 6 mM $HAuCl_4$ was placed on the printed array chip covering all working electrodes. The chip was connected to Autolab potentiostat (Metrohm, Netherlands) operated by NOVA 1.9 software, and then chronoamperometry was applied at a potential of -0.85 V over a period of 200 seconds for each working electrode until the surface turns visibly golden (figure A.1). Chips were then washed with deionized water and dried then stored for further use.

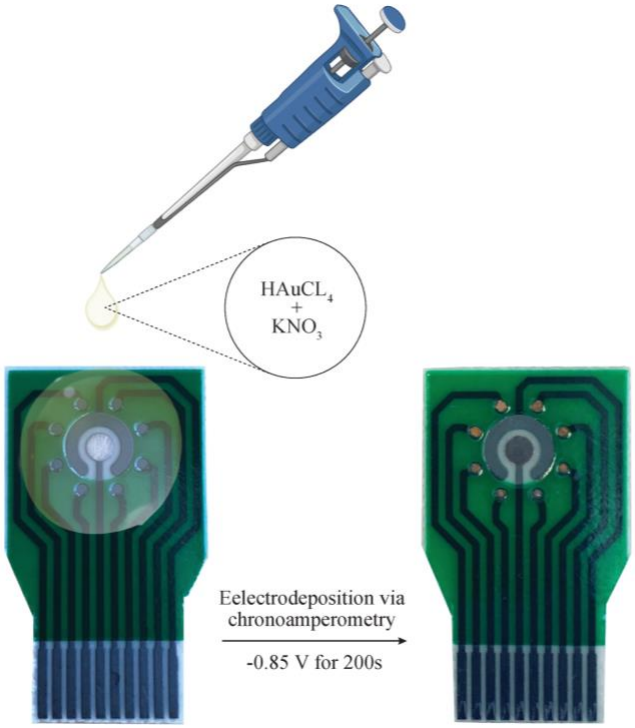

Figure A.1 Array chip before and after electrodeposition with $HAuCl_4$ via chronoamperometry.

A.2 Optimization of AuNP electrodeposition

In immunosensors development, improving the sensitivity can be achieved by increasing the surface area of the transducer to allow more antibodies to bind to the surface, which in turn enhances the sensitivity and gives a further accurate data. For that reason, among others, electrodeposition of AuNPs on the working electrode (transducer) is extensively used in developing biosensors. Various electrochemical methods are used like cyclic voltammetry (CV) and chronoamperometry. Chronoamperometry uses potential applied to working electrode, and measures current as a function of time (Guy et Walker, 2016). Electrodeposition of Au particles on carbon printed electrode via the reduction of KNO_3 solution of $HAuCl_4$ by chronoamperometry was done using the previously stated parameters. However, tuning and optimizing deposition time, deposition potential and gold solution concentration is possible, as it can result in different morphologies of Au particles.

First, one CV was performed, and three points were selected on the reduction peak of the voltammogram as shown in Figure A.2. Reduction started at potential of -0.9 V, therefore, three points were selected; prior to, after and in the middle of the reduction peak (-0.85 V, -0.95 V and -1.1 V). The potential -0.85 V was selected first for electrodeposition of AuNP via chronoamperometry, and different deposition time periods were tried with the first potential (50 s, 100 s, and 200 s). SWV was measured after each deposition, to obtain the current (μA) for each time period used. The highest current achieved was when electrodepositing $HAuCl_4$ at -0.85 V for 200 s (Figure A.3). Scanning electron microscope (SEM) images were taken for electrodeposited carbon electrode at potential -0.85 V for 200 s (Figure A.4).

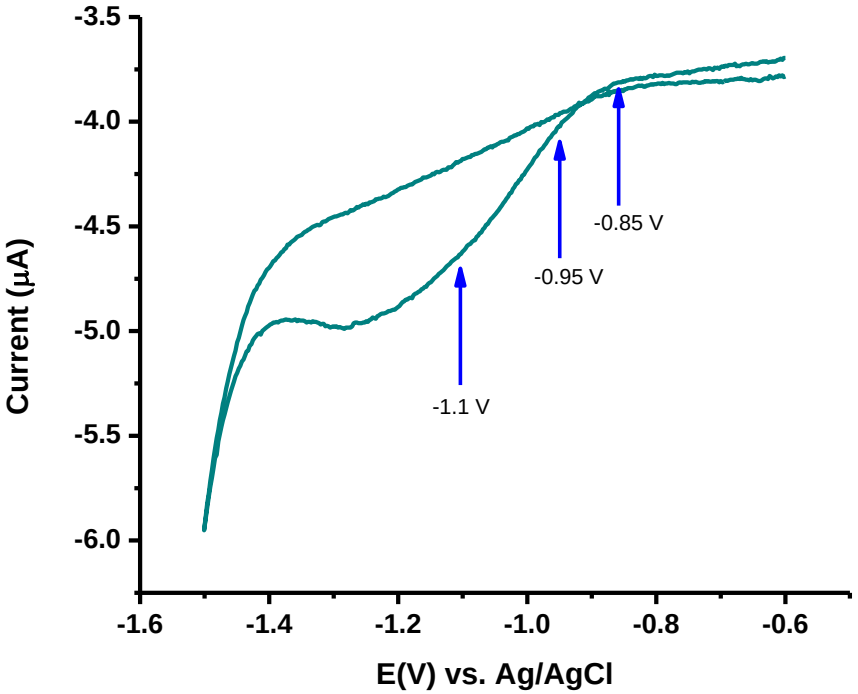

Figure A.2 Cyclic voltammogram shows reduction starting at -0.9 V and the three chosen potentials.

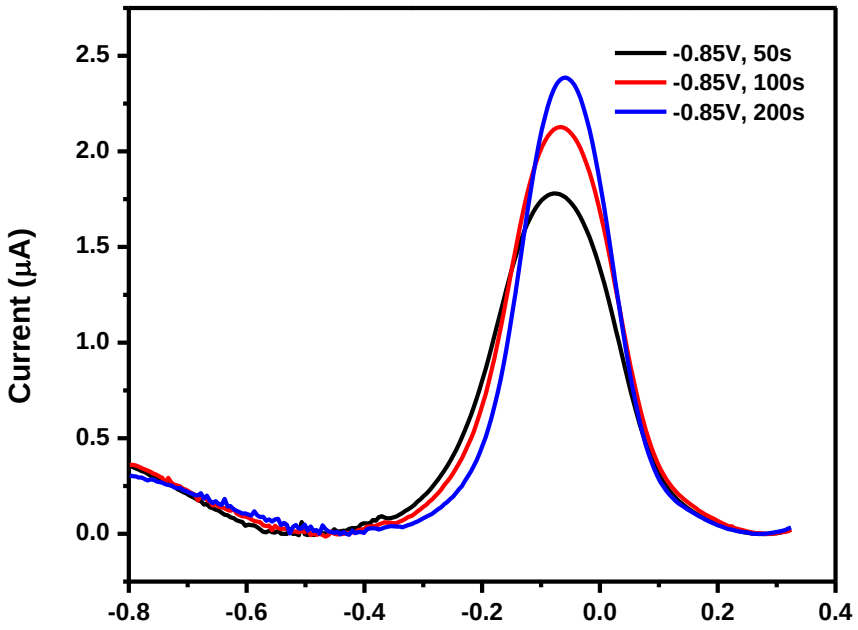

Figure A.3 SWV measured after electrodeposition of AuNPs via chronoamperometry of different time periods of the same potential (-0.85 V) for (50 s, 100 s and 200 s).

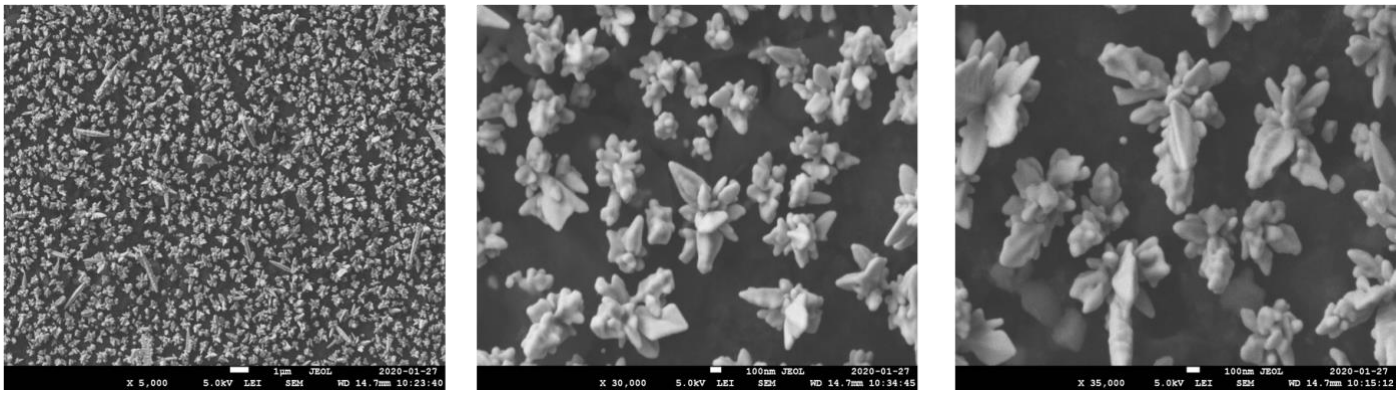

Figure A.4 Scanning electron microscope (SEM) images at different magnifications of Au-deposited carbon electrode via chronoamperometry at -0.85 V for 200 s.

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