DEVELOPMENT OF AN SPR-BASED BIOSENSOR APPLICABLE TO THE RAPID AND EARLY DETECTION OF DISEASE BIOMARKERS

by

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AUTHOR'S DECLARATION

I, Peerawich Sukcharoenchaikul, declare that the research work carried out for this thesis was in accordance with the regulations of the Asian Institute of Technology. The work presented in it are my own and has been generated by me as the result of my own original research, and if external sources were used, such sources have been cited. It is original and has not been submitted to any other institution to obtain another degree or qualification. This is a true copy of the thesis, including final revisions.

Date: 25/07/2022

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Signature: 

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ABSTRACT

From centuries, infectious diseases have been one of the main factors influencing human's mortality rate, thus disease's detection is crucial to keep it low. It has been proved that early detection of diseases can potentially ease the severity and increases precise diagnosis for proper treatment. This research will be focused on stimulating biosensor usage by applying an optical biosensor in detecting glycated albumin which is a mockup of a biomarker. By using an optical biosensor applied with surface plasmon resonance (SPR) in detecting glycated albumin in order to analyze SPR peak shift of gold particles that deposit on ZnO nanorods which have aptamers immobilization on the gold surface. In this research, the biosensor has been used to evaluate glycated albumin at several concentrations and it has been found that the glycated albumin could be rapidly analyzed that the biosensor can detect. In future implications, this research could be potentially further developed to be used with a biomarker that is a real infectious disease to increase the disease's detection preciseness and speediness.

Keyword: biosensor, surface plasmon resonance, SPR, biomarker, glycated albumin

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LIST OF ABBREVIATIONS

AIDS	= Acquired Immunodeficiency Syndrome
COVID-19	= Coronavirus Disease
DFA	= Direct Immunofluorescence Assay
DNA	= Deoxyribonucleic Acid
EIA	= Enzyme Immunoassay
ELISA	= Enzyme-Linked Immunosorbent Assay
H1N1	= Influenza A Virus Subtype H1N1
HIV	= Human Immunodeficiency Viruses
IEP	= Isoelectric Point
IF	= Immunofluorescence
IFA	= Immunofluorescence Assays
LAMP	= Loop-Mediated Isothermal Amplification
LOD	= Limit Of Detection
LSPR	= Localized Surface Plasmon Resonance
MERS	= Middle East Respiratory Syndrome
NPs	= Nanoparticle
PCR	= Polymerase Chain Reaction
POC	= Point Of Care
RNA	= Ribonucleic Acid
RT-LAMP	= Reverse Transcription Loop-Mediated Isothermal Amplification
RT-PCR	= Reverse Transcription Polymerase Chain Reaction
SAMs	= Self-Assembled Monolayers
SELEX	= Systematic Evolution Of Ligands By Exponential Enrichment
SEM	= Scanning Electron Microscopy
SP	= Surface Plasmon
SPR	= Surface Plasmon Resonance
UV	= Ultraviolet

CHAPTER 1
INTRODUCTION

1.1 Background of the Study

Viral diseases are concerning for human health as many pathogenic agents are continuously changing their genetic composition to evade the host immune system (Liu et al., 2015; Abid et al., 2021).

In recent times, disease outbreaks and epidemics have occurred across the world as a result of more than 20 infectious agents (A. A. Balkhair, 2020). H1N1 and MERS are some examples of outbreaks of infectious pathogens. COVID-19 is one of the most recent outbreaks that are caused by a mutation of a single-stranded RNA coronavirus (Asif et al., 2020). Changing landscapes of local environments have made RNA viruses to rapidly adapt their genetic material to these diversifying conditions because of higher replication rate errors (Nichol et al., 2000).

As a result, researchers have been working intensively to uncover the probable origins of the virus and provide several therapeutic medications, vaccinations, and diagnoses about infectious illnesses that have been obtained and continue to be learned to help detect the viral diseases before it gets out of control (Morens & Fauci, 2020). Vaccinations and current technologies have drastically reduced the mortality rate caused by these disease mutations so far (Fung, 2012).

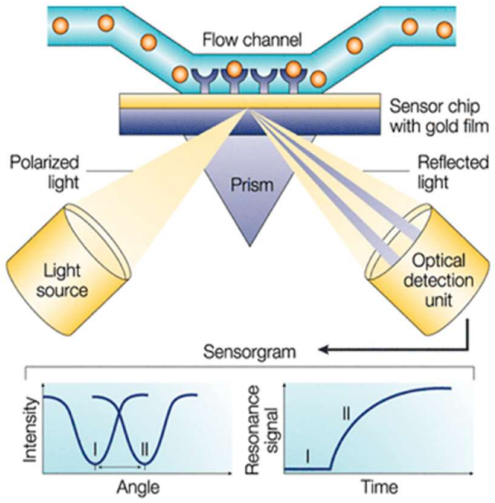
Currently, the detection of several biomarkers can be divided into two categories which are direct detection methods and indirect detection methods (Carter et al., 2020). Direct detection methods, also known as molecular diagnostic techniques, are based on the specific nucleic acid sequence detection that can provide a more rapid, sensitive, and specific detection than traditional diagnostic (Reta et al., 2020). The nucleic acid amplification assay of polymerase chain reaction (PCR) is one example of a direct detection method. Other direct detection techniques are Reverse Transcription-PCR (RT-PCR) and Loop-Mediated Isothermal Amplification (LAMP) (Cui & Zhou, 2020).

On the other hand, indirect detection methods, divided into serological and immunological assays, related to the detection in blood serum or in plasma (Carter et al., 2020), also use disease-specific antibodies (Ji et al., 2020). Examples of serological and immunological assays are Enzyme-Linked Immunosorbent Assay (ELISA), Lateral Flow Immunoassay, and Immunofluorescence (IF) assay (Giri et al., 2021).

Biosensors can be made from a variety of transducers. The optical transducer is one of the most significant due to its high sensitivity, as a result of developments that have improved the signal-to-noise ratio and lowered the detection limit (Bhalla et al., 2016; Damborský et al., 2016). One of the most remarkable optical transducers is the SPR biosensor. Surface Plasmon Resonance (SPR) is used to detect the binding between an interesting molecule (analyte) and biorecognition (ligand) which does not require any additional labels (Piliarik & Homola, 2009). This phenomenon appears when the beam of light hits the surface of the material which mostly is gold (Mullett et al., 2000). The electron is transferred due to the excitation of the electron in the material interacting with the light and becoming a plasmon (Nguyen et al., 2015). Glass prism is used to make reflected light and the excitation between the electron and the light makes the angle of the define SPR (resonance) occurred which relies on the refractive index of the material (Gosu & Zaheer, 2021). When the biomolecule binds with the biorecognition, the reflective index will be changed and can be detected by measuring the shift of resonance angle which can be seen in Figure 1.1 (Chauhan & Kumar Singh, 2021). Additionally, an analyte used with the biosensor, glycated albumin is a biomarker that could be related to several pathogens (Arasteh et al., 2014). It could be used instead of actual infectious pathogen samples because their mass is relatively comparable and can be used to demonstrate the detection of real-world diseases such as diabetes (Freitas et al., 2017).

Figure 1.1

SPR Principle



Note. The concept of the surface plasmon resonance method measures the angle shift of the surface plasmon caused by the binding of the receptor and substrate (Pires et al., 2014).

1.2 Statement of the Problem

New diseases are on the rise throughout the world, and this poses a difficulty in identifying and diagnosing them, due to a lack of identification of the biomarkers that can be accurately detected in patients (Warren et al., 2014). There is thus the need for diagnostic technologies that can not only effectively identify diseases but also provide accurate treatment recommendations to avoid their transmission (Mavrikou et al., 2020).

The new methods which able to detect biomarkers can be used in point of care (POC) devices which are the consequence of advanced biosensors (Harpaz et al., 2019). This development leads to faster and easier detection (Syedmoradi et al., 2021). Several POC biosensors can be used to detect biomarkers such as electrochemical, thermometric, piezoelectric, and optic (Anik, 2017). For the precise detection of a biomarker utilizing the optical biosensor, an SPR POC device, have the ability to provide results in quick

detection (Harpaz et al., 2019). Optical SPR biosensors are significant devices for molecular interactions (Guo, 2012). For compounds with molecular weights greater than 1000 Da, the SPR biosensor has a detection limit of 10 pg/mL (Nguyen et al., 2015). Furthermore, adding some label proteins to the analyte of the surface can boost sensitivity; this is known as SPR signal amplification (Chung et al., 2005).

SPR biosensor methodology utilizes a system capable of identifying in real-time biomarkers that lead to infectious illness, such as SARS-CoV-2, and other antigens (Djaileb et al., 2020). SPR biosensing can improve the efficiency of the detection by exploiting the aptamer to bind with the biomarker (Nguyen et al., 2015). Additionally, traditional detection methods are often time-consuming and expensive. For example, it takes up to 4 hours to perform one experiment of RT-PCR. Instead, SPR-based techniques utilizing aptamers can detect several biomarkers more accurately and in a shorter time (Samson et al., 2020).

Currently, advancements in the field of nanotechnology are increasing as well as the application of surface plasmons for specialized applications which emerged as a potential alternative for traditional optical sensors (Petryayeva & Krull, 2011). The growth of this nanotechnology application on optical sensors has also resulted in the development of localized surface plasmon resonance (LSPR) (Kosuda et al., 2019). The difference between SPR and LSPR is the SPR interacts in a thin metallic layer where electromagnetic wave spreads at the surface which required a complex instrument to perform and LSPR oscillates when incident light interacts in metal nanoparticles like gold nanoparticles due to the confinement of the electromagnetic field which is easy to perform by measure color change and absorption peak shift (Damborský et al., 2016). LSPR commonly occurs usually with metallic which are smaller than the wavelength of the incident light (Petryayeva & Krull, 2011). Conduction electrons oscillate (surface plasmons) is the result of the fluctuating electric field that is produced when incident light interacts with gold or silver nanoparticles (Paul & Giri, 2018). Since this phenomenon is dependent on variations in resonant peak shift output, these methods are customizable and can be employed in a biosensor by modifying a few of the materials, thicknesses, and sizes of the nanostructures that are suitable for LSPR (Chauhan & Kumar Singh, 2021).

1.3 Objectives of the Research

The principal aim of the research will be to design an SPR biosensor that detects biomarkers related to dangerous diseases utilizing an aptamers recognition system. In this project, a biosensor functionalized on a glass substrate will be used to identify glycated albumin as a model analyte. The objectives are as follows:

1. Synthesis of a biosensor with SPR capabilities for the detection of a model protein via aptamer binding. Here glycated albumin will be used.
2. Surface functionalization of substrates like glass and assessment of the capability for glycated albumin detection.
3. Data collection and analysis on its effectiveness in detecting different quantities of glycated albumin.

1.4 Scope of the Study

The focus of this study will be on SPR-based biosensors for some biomarkers such as glycated albumin detection (Sasar et al., 2020). The chosen aptamer will be utilized to interact with varying concentrations of glycated albumin, after its immobilization aptamer on a ZnO nanorods substrate. The results of the binding between the aptamer and the biomarker, as well as the signal output, will be examined utilizing a range of choices available to assess the biosensor effectiveness.

1.5 Limitation of the Study

1. This study detects a specific model protein, namely glycated albumin, as a biomarker. Real-world biomarkers are not included.
2. The sensor transducer will only be Surface Plasmon Resonance (SPR) based. Other biosensors are not included to be investigated.
3. The sensor's base is made from ZnO nanorods on a glass substrate. Other morphologies of ZnO are not taken into account.

CHAPTER 2
LITERATURE REVIEW

2.1 Biosensor State of Arts

2.1.1 Biosensors Working Principle

A biosensor is a type of sensor that can analyze biological substrates such as microorganisms or chemical interactions (Guo, 2012). The simple principle of the biosensor is to convert the signal that it receives from the bioreceptor which is immobilized on the surface of the transducer material to a detectable signal, the signal's output will then be analyzed and changed to a readable signal (Bhalla et al., 2016).

Biosensors are divided into two parts: biosensors based on biological signals and biosensors based on transducers (Perumal & Hashim, 2014). Biosensors based on a biological signal's main feature is the bioreceptor, often known as the biological recognition element and it must be selective and sensitive to the specific target analyte (Sawant, 2017). On the other hand, biosensors based on transducers are categorized on the transduction method that has been used to convert the change of physical and chemical into the electrical signal (Lowe, 2008).

In biosensors based on a biological signal, the biological recognition element is classified into five types of sensors: enzyme-based sensors, immunosensors, DNA/nucleic acid sensors, cell-based sensors, and biomimetic sensors (Perumal & Hashim, 2014).

The biosensor based on transducers is classified based on the mechanism of transmission they use (Naresh & Lee, 2021). The transducer is a significant component to transfer the energy to signal detection such as the electrochemical sensor, optical biosensors, and piezoelectric sensor (Liu et al., 2018).

2.1.2 Classification of Biosensor Base on Transducers

Classification of transducers are generically classified as electrochemical, optical, and piezoelectric sensors based on the operating principles (Naresh & Lee, 2021).

For the example of a biosensor based on transducers, the electrochemical sensor is used electrodes to be a transducer and analyze values that receive from different measurements including amperometric and potentiometric biosensors (Perumal & Hashim, 2014). Amperometric biosensors monitor the product current during the oxidation or reduction of the process while potentiometric biosensors measure the potential difference between the biosensor electrode and the reference electrode (Sawant, 2017).

Optical biosensors work by combining optical characteristics with a biorecognition component to create a label or label-free detection (Guo, 2012). Several analytes can be used to measure by optical spectroscopy such as absorption, Raman, surface plasmon resonance (SPR), localized surface plasmon resonance (LSPR), and fluorescence (Damborský et al., 2016). Surface plasmon resonance (SPR) is known to be the most sophisticated technology for understanding biomolecular interactions (Piliarik & Homola, 2009). SPR has since improved surface chemistry, helping in the advancement of chemistry, physics, and biology (Singh, 2016). The SPR detection limit is as low as a single molecule whose detection range is between μM and nM, making it very sensitive (Guo, 2012).

Nevertheless, The SPR technique does have certain disadvantages. The main disadvantage is the system's sensitivity, with a limit of detection defined at 1-10nM for a 20 kDa protein especially when the binding has a low affinity or the surface has a limited binding capacity which makes it difficult for detection (Mustafa et al., 2010).

In a piezoelectric sensor, the mechanical or electrical changes that result from the piezoelectric effect from piezoelectric crystals often convert to electrical signals (Liu et al., 2018). This signal was detected by the sensors and the characteristics of the crystals elastic generally define the signal output (Srivastava et al., 2020).

2.1.3 Biosensors Characteristics

One of the most essential characteristics of biosensors is selectivity, as it is the ability of the receptor that can detect specific analyses (Akgönüllü et al., 2020).

In addition, sensitivity is the lowest level of analysis that biosensors can detect, and it is related to the limit of detection (LOD) (Abid et al., 2021). The biosensor should be able to recognize substrate concentrations as low as possible in order to verify the existence of substrate residues inside the samples. (Liu et al., 2018).

Moreover, reproducibility is also crucial within the biosensor, as it is the ability to reproduce the experiment with precision and accurate responses for the reliability of information (Bhalla et al., 2016).

Lastly, the significant characteristic of biosensors is linearity. Linearity is the ability that shows the characteristic indicating the precision of the measured response and it is affected by its resolution (Naresh & Lee, 2021).

2.1.4 Biosensor Components

Analytes, receptors, transducers, electronics, and displays are the five components of a biosensor (see Figure 2.1) and they can be divided depending on the physical signal that sensors are produced such as electrochemical sensors, optical sensors, and thermal sensors (Kheyraddini Mousavi et al., 2012; Bhalla et al., 2016). Biosensors are a combination of biological and electronic components that lead to the efficiency of detection (Sawant, 2017).

For the components of the biosensor, the bioreceptor is a biochemical receptor with a recognition component that functions as an interactor to the target analyte such as aptamers, enzymes, antibodies, and deoxyribonucleic acid (DNA) (Naresh & Lee, 2021). Therefore, the selectivity and sensitivity of the system rely mainly on the biorecognition-specific target due to its ability to bind the target (Bhalla et al., 2016). However, some external factors can affect the biorecognition component including variables of temperature, pH, and ionic strength. (Sawant, 2017).

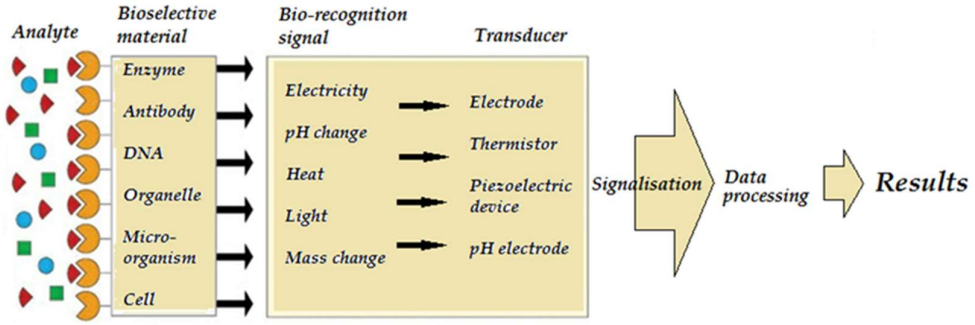
The transducer is a converter that changes in terms of chemical energy to another energy for analysis such as electrical signals (Perumal & Hashim, 2014; Bhalla et al., 2016). When a bioreceptor binds to the analyte, the chemical or physical reaction occurs, and the transducer detects the appearing reaction and changes to some biochemical signal

or quantifiable electronic signal (Fatoyinbo & Hughes, 2012). The signal is then sent to the processing section which amplifies the signal before sending (Naresh & Lee, 2021).

When the signal is generated by the transducer, a signal processor (electronic parts) receives the signal and converts the transducer's signal into a readable signal for analysis (Kheyraddini Mousavi et al., 2012). The display section displays the signal's interpretation using a liquid crystal display and a computer to present the comprehended information (Bhalla et al., 2016).

Figure 2.1

Biosensor's Mechanism



Note. The biosensor's mechanism, which includes analyte, biorecognition, transducer, and signal processing, is demonstrated in this figure (Kivirand et al., 2013).

2.1.5 Biosensors for Infectious Disease

Biomarkers, biomolecules, and biomaterials have been used to determine the presence or absence of infectious disease for a long time which can be applied to the use of biosensors (Firdous et al., 2018). Electrochemical biosensors could be a commonly used approach to detect infectious diseases due to their easy procedure, good selectivity, and capability to perform a portable device (Sheikhzadeh et al., 2016). Furthermore, this technique enables the mass production of portable and low-cost analytical equipment that can operate with very small sample quantities (Eissa et al., 2018).

Furthermore, optical biosensors also performed an advanced and important method for analyzing molecular interactions which can lead to infectious disease (Mullett et al., 2000). They provide fast detection with excellent sensitivity and selectivity, as well as simple and uncomplicated detection (Guo, 2012). Surface Plasmon Resonance (SPR) biosensors are some of the optical biosensors and are alternative to antibody-based methods which have been applied to detect and quantify analytes from biomolecules such as proteins, nucleic acids, and small molecules, including drugs and their metabolites (Mehrotra, 2016). SPR biosensor technology has been used successfully for the detection of infectious disease markers such as HIV-1 p24 protein, Human Immunodeficiency Virus (HIV) antibodies IgG/IgM antibody detection in blood samples, HBsAg hepatitis B virus antigen detection in serum samples (Ozer & Henry, 2021).

These aforementioned biosensors can detect symptoms on a molecular level and can detect symptoms as soon as they appear, which means the diagnosis is easier and faster than ever before with the use of biosensors (Cui & Zhou, 2020). These biosensors have been providing more accurate medical diagnoses of infectious diseases, increasing treatment possibilities (Abid et al., 2021). In addition, the invention of biosensors has accelerated the process for the medical diagnosis of infectious diseases (Sin et al., 2014). These devices are capable of detecting pathogens in blood, saliva, urine, and other bodily fluids (Sin et al., 2014). The interaction among antigen-antibodies has been employed in the clinical diagnosis of infectious diseases and other conditions (Chung et al., 2005). If an illness is to be properly treated, it must be detected as early as possible (Yakoh et al., 2021).

2.2 Current Assays to Detect Viral Antigens (e.g.: SARS-CoV-2 RBD)

An infectious disease is a disease that is caused by an infectious agent, which is transmitted into the body by an infected host (Cinti et al., 2008). Some of the most common infectious diseases are influenza (flu), HIV/AIDS, hepatitis A and B, malaria, syphilis, and Zika virus infection (Holmes et al., 2017). These types of diseases are caused by microorganisms such as bacteria, viruses, fungi, or parasites (Cole & Kramer, 2016). Infectious diseases can spread through the air, food, water, or contact with an infected person or animal (Cinti et al., 2008). The consequences of infectious diseases

are serious because they can lead to death and some diseases also lead to permanent damage to the body, and they can infect individuals, while others cause discomforts, such as cold or flu (Souf, 2016). As a result, the detection of these diseases or virology diagnostics is critical for treating and monitoring the symptoms of these illnesses (Trent, 2005).

Diagnostic virology is the section of clinical medicine that assesses individuals to diagnose, treat, and prevent illness as a result of the convergence of several independent disease developments (Storch, 2000). Technical advances, especially in the field of nucleic acid chemistry have erupted in the creation of crucial new instruments for viral diagnostics (Storch, 2000).

Previously, laboratory diagnosis of viruses has involved isolating infections in tissue culture or viral detection methods based on cell culture as well as visual inspection of pathogens in the sample using electron microscopy and other techniques (Cella et al., 2013). There are certain notable advantages of viral culture, including the ability to identify numerous viruses, even if not all of them were suspected at the time the culture was conducted (Storch, 2000). However, there are certain drawbacks, such as being time-consuming, costly, and difficult to replicate (Abid et al., 2021).

Molecular techniques have altered diagnostic infectious diseases since they determine the presence and disappearance of viral nucleic acids in a patient's sample (Acharya et al., 2004). Thus, numerous conventional techniques have been replaced by approaches based on nucleic acids (Atmar, 2014). Rapid and precise disease identification enhances the chances of living, lowers the cost of ineffective therapies, and prevents disease transmission (Acharya et al., 2004).

For the molecular techniques (direct diagnostic procedures), molecular methods which include the multiplication of viral genomic information are very sensitive and selective, enable fast diagnosis, and provide the simultaneous detection of several viruses (Cella et al., 2013). Molecular detection methods examine the nucleic acids in the sample to determine the virus and this technique has been used to diagnose and track the number of different viral illnesses (Giri et al., 2021).

Polymerase Chain Reaction (PCR) is a well-known nucleic acid amplification technique and is typically used to detect or identify viruses (Emery et al., 2004). PCR can also be used to detect viruses in a wide range of specimen types (Yakoh et al., 2021). PCR utilizes a thermostable of DNA polymerase and two oligonucleotide primers to extract and purify DNA molecules, followed by exponential amplification of the target sequence (Jackson, 1990). Numerous procedures can also be used to evaluate the amplified result after the PCR process (Atmar, 2014). The notable technique often used are reverse transcription-PCR and real-time PCR (Reta et al., 2020). However, traditional PCR is still in use in several clinical laboratories across the world, although it is being phased out in favor of more sophisticated PCR versions (Jackson, 1990).

Real-time PCR and reverse transcription-PCR are the most frequently utilized types of conventional amplification (Souf, 2016). Both are currently considered standards for determining infection rates (Atmar, 2014). The concept of RT-PCR is to use reverse transcriptase to modify the virus's RNA and convert it to complementary DNA (cDNA) (Bustin et al., 2005; Carter et al., 2020).

Furthermore, the cDNA is utilized as a template for amplification using PCR to measure gene expression (Atmar, 2014). Depending on the origin substance, the RT-PCR test is feasible to be done in one or two stages, with one step getting quicker results than two steps (Giri et al., 2021). Before the DNA polymerase process, the RNA template must be reverse transcribed in step two because RNA must be transcribed to DNA to be amplified while one step uses only a single tube to analyze (Yakoh et al., 2021).

One-step real-time RT-PCR performs the whole RT-PCR reaction in a single tube containing sufficient primers (Carter et al., 2020). The two-step technique involves RNA in one tube followed by DNA polymerization through another (Giri et al., 2021). RT-PCR has been used to diagnose human infection by RNA viruses since the beginning (Reta et al., 2020).

However, due to current methods not providing enough rapid and cost-effective testing, reverse transcription loop-mediated isothermal amplification (RT-LAMP) was

developed for the alternative method (Carter et al., 2020). The viral RNA and DNA are being tested using this approach (Reta et al., 2020).

A standard RT-qPCR procedure requires around 90-120 minutes to complete, while LAMP takes about half an hour to complete (Kubina & Dziedzic, 2020). Four to six unique primers specific to the target gene/region were employed to enhance sensitivity, and this approach was combined with the reverse transcription procedure to detect viral RNA or bacteria (Carter et al., 2020).

RT-LAMP is the technique of amplifying the RNA sequence to improve specificity, sensitivity, and detection time (Courrol et al., 2007). The thermal cycle is not used in this approach (Samson et al., 2020). Since it is an isothermal condition, this technique will produce results faster than RT-PCR (Fu et al., 2011). The presence of certain RNA samples could be detected using RT-LAMP (Carter et al., 2020). To detect the viral RNA, the exact sequencing of the virus is required to determine the result (Kubina & Dziedzic, 2020). Therefore, the detection can be completed by comparing the samples' sequences to a known sequence to identify the individual virus (Fu et al., 2011).

The detection of viral nucleic acids is considered to be the most reliable method for identifying viral infection (Kubina & Dziedzic, 2020). On the other hand, it has several limitations, including the patient's previous infection and immunity, and it takes longer and requires more effort in analyzing the virus (Fu et al., 2011). As a result, serological testing can overcome the limitations of molecular assays (Carter et al., 2020). This approach identifies the patient's antigen and antibody to recognize the virus by scanning for antibodies (IgG and IgM) in the blood, saliva, and sputum (Giri et al., 2021).

Immunological techniques (indirect diagnostic procedures) have been used for detection for centuries (Atmar, 2014). For the identification of human viral infections, several immunological diagnostic options are proposed (Reta et al., 2020). The body's immune system produces antibodies to interact with viral infections, and this interaction was used to develop immunological diagnostic methods (Storch, 2000). There are many approaches to identifying viral infection in patient specimens, including numerous immunological diagnostic methods, such as ELISA and Immunofluorescence (IF) assays (Reta et al., 2020).

Enzyme-Linked Immunosorbent Assay (ELISA) or Enzyme Immunoassay (EIA) is the technique that detects the presence of viral antigen by utilizing antibodies that are specific to the antigen (Kubina & Dziedzic, 2020). ELISA tests are used for various purposes such as detecting drugs or determining blood type and it is a powerful tool for the detection of antibodies in both clinical and research settings (Aydin, 2015).

The basic principle of an ELISA is straightforward (Reta et al., 2020). The detection could be performed by knowing the number of antibodies that bind to the antigen, it will form a complex that can be detected by the addition of an enzyme-labeled antibody or another reporter molecule to form an enzymatic reaction product (Shah & Maghsoudlou, 2016). However, this method can only detect the presence and disappearance of viral antigen, but it cannot determine the sample's depth to exact concentration (De La Rica & Stevens, 2012).

There are four major types of ELISA (see Figure 2.2): direct ELISA, indirect ELISA, antigen-specific antibodies sandwich ELISA, and competitive ELISA (Shah & Maghsoudlou, 2016). Direct ELISA is an enzyme-linked immunosorbent assay that uses antigen-specific antibodies to detect antibody molecules (Lalani et al., 2018). It does not use any other reagents or chemicals to amplify the signal (Reta et al., 2020).

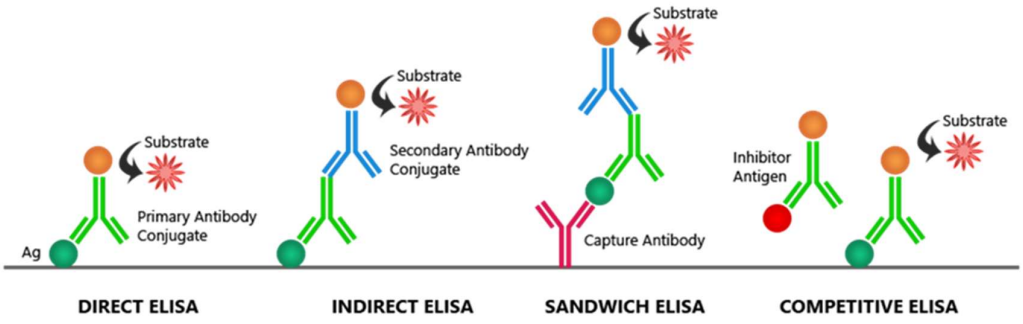
On the other hand, indirect ELISA is a technique that determines the presence of antibodies in an unknown sample by using the known concentrations of antigens in a standard sample (Shah & Maghsoudlou, 2016).

Sandwich ELISA has become the most popular type of ELISA for biomedical research, and it contrasts with direct and indirect ELISA because it can use two different antibodies (Louten, 2016). One of which binds to an antigen on the test sample which binds to and selectively labels and the other one binds to antibodies that are placed onto a solid matrix (Kubina & Dziedzic, 2020).

In competitive ELISA, the antigen is immobilized on a well plate, and an unlabeled primary antibody is added to detect the antigen (Shah & Maghsoudlou, 2016). This assay provides very sensitive detection of antibodies by adding an excess amount of unlabeled antibodies to compete with an analyte for binding sites and it can detect very low levels of antibodies in a sample (Lalani et al., 2018).

Figure 2.2

Different Forms of ELISA



Note. The different forms of ELISA are depicted in this figure, which is determined by the antibodies and techniques employed (BOSTER, n.d.).

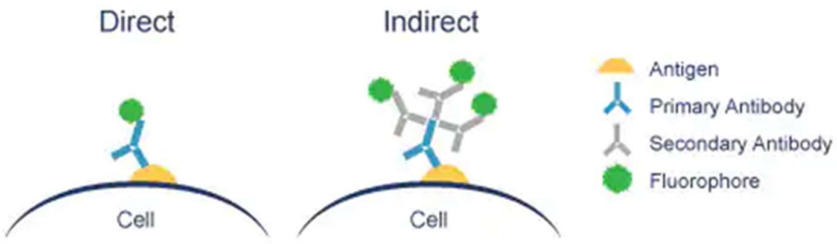
Immunofluorescence (IF) assays are one of the most used tests in medical diagnosis (Reta et al., 2020). The immunofluorescence assay is a technique that works by detecting the presence of a particular antigen and antibodies (Atmar, 2014). This approach can be divided into two types which are direct (DFA) and indirect immunofluorescence assays (IFA) (see Figure 2.3) (Reta et al., 2020).

The direct immunofluorescence assay (DFA) technique involves the use of antibodies that bind to specific antigens and then get labeled with a fluorescent dye, or as known as a fluorophore-conjugated antibody (Louten, 2016). The fluorescently labeled antibodies are then applied to the sample, where they bind to any antigens present and emit light for detection (Louten, 2016).

Indirect immunofluorescence assays (IFA), instead of fluorescence, the enzyme binds to the antibody, which is an unlabeled specific antibody and employs two antibodies instead of one (Reta et al., 2020). The use of an unlabeled antibody that can bind to a specific viral antigen becomes the first method (Kubina & Dziedzic, 2020). Then, if a response occurs from the first antibody binding with an attached fluorescent-tagged antibody, it can be detected (Atmar, 2014).

Figure 2.3

Immunofluorescence (IF) Assays



Note. The figure demonstrates both direct and indirect Immunofluorescence Techniques. These approaches have benefits and drawbacks which are dependent on how individual detection is employed (Abcam, n.d.).

2.3 Surface Functionalization of Biorecognition on SPR-based Sensors

2.3.1 Immobilization of the Biorecognition on the Surface

Due to the sensor needing a high density of biorecognition elements on the surface while preserving biological processes, surface chemistries were used for biorecognition element immobilization on the SPR and there is no conventional procedure for immobilization (Balamurugan et al., 2008). As a result, the immobilization chemistry is chosen depending on the characteristics of a certain biorecognition element (Piliarik et al., 2009).

Several varieties of biorecognition elements and immobilization techniques are sufficient to use with biosensors for customized specific detection (Guo, 2012). Antibodies, peptides, and aptamers are examples of biorecognition elements that can be employed to immobilize the SPR surface (Piliarik et al., 2009).

Maintaining the bonding sensitivity and selectivity of the aptamer in solution is an essential aim for biomolecules immobilization (Firdous et al., 2018). Several methods for immobilizing biomolecules on the SPR sensor surface have already been discovered

such as physical adsorption techniques based on hydrophobic, strong covalent bonding, non-covalent interaction of the tag proteins, and a target molecule (Löfås et al., 1995).

Initially, SPR applications relied on physic adsorption to a protein since it is simple and straightforward, such as Van der Waals interactions (Löfås & Mcwhirter, 2006). However, since complicated techniques or structures of biorecognition are constantly being developed, simple approvals to immobilize bio recognition are insufficient (Balamurugan et al., 2008). Therefore, the spontaneous adsorption of proteins with other molecules, typically utilizing silver and gold, is commonly used, followed by passive attachment to a metal substrate (Löfås & Mcwhirter, 2006). The electrostatic and Van der Waals interactions provides the most convenient and low-cost approach, but it has poor binding stability (Kozitsina et al., 2018). The physic adsorption technique has weaker bonding than the other techniques, but it does not modify the structure of the immobilized natural protein (Jesionowski et al., 2014).

Covalent bonding is one of the most widely used methods and it provides a strong binding between biorecognition and the surface substrate (Martinkova et al., 2017). A covalent bond form can be a stable way to form a protein immobilized on the surface sensor by combining usable functional groups of exposure amino acids, such as amine groups and thiol groups, with appropriate forms of functionalized surfaces, such as carboxylic acid, aldehyde, maleimide, and vinyl sulfone (Tomizaki et al., 2005). This method of immobilizing biorecognition has the advantage of being a strong stable and reliable immobilization method (Homola, 2008).

2.3.2 Surfaces Capable of Immobilizing Biorecognition

Glass and polymer substrates are commonly used to avoid signal extinguishment for optical measurements and the clarity of the signal (Piliarik & Homola, 2009). It allows signal transmission and eliminates spectroscopic extinguishment that may occur with a gold surface (Balamurugan et al., 2008). In addition, ZnO has been employed in a variety of applications since they have several advantages such as it can easily be functionalized and modified with different functional groups and also offers the advantage of high specificity and stability (Yaqoob et al., 2020). ZnO is anticipated to be an outstanding electrochemical material with a low isoelectric point (IEP), enabling

it to be much more beneficial in solar cell manufacturing and semiconductor material (Rajendra et al., 2014).

ZnO substrate was used to be the substrate for immobilization of several proteins such as enzymes, antibodies, and aptamers due to it can enhance the performance of the sensor (Ahmad et al., 2014). ZnO can be applied to different substrates, and it can be grown for use to be an electrode which has to prepare some additional methods (Ahmad et al., 2014). Moreover, ZnO has a high capacity for absorption, high specific surface area, and remarkable optical transparency over a wide range of wavelengths, including visible and near-infrared wavelengths making it potential material for immobilization of biomolecules (Ahmad et al., 2014). As a result, in order to produce ZnO, ZnO can be hydrothermally grown on a glass substrate to improve the sensor's efficiency (Li et al., 2019). Moreover, nanorods are regarded as one of the greatest opportunities for future integrated biosensors among oxide semiconductors due to their exceptional electrical properties and biocompatibility (Rajendra et al., 2014).

2.3.3 Functionalization of Aptamer on the Gold Surface

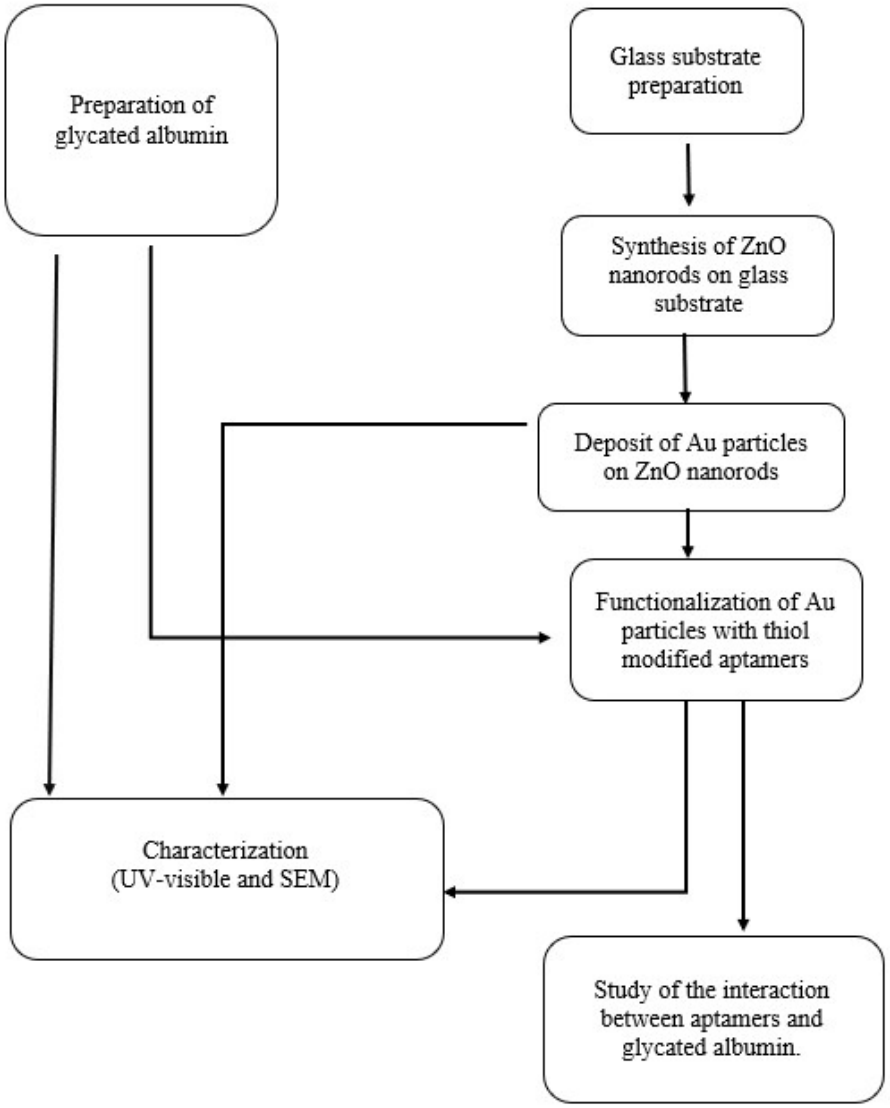
For the direct attachment of aptamers on the gold surface, DNA linked by the thiol-terminated side has been discovered during DNA hybridization investigations previously (Balamurugan et al., 2008). Methods for binding aptamers on gold surfaces are similar to what is used to link nonbiological monolayers produced by thiol chemisorption with gold substrates (Baldrich et al., 2005).

An aptamer's immobilization on a gold surface can be achieved by employing an alkanethiol self-assembly method attached to an aptamer sequence through chemisorption (Herne & Tarlov, 1997). For aptamers immobilization on a monolayer gold surface, thiol-terminated aptamers typically were used and comprise three components which are a thiol (–SH) or disulfide (–SSR) terminus, a linker, and the aptamers sequence (Thiviyanathan & Gorenstein, 2012). Therefore, the terminal thiol group binds to the aptamers towards the gold surface (Löfås & Mcwhirter, 2006). The variety of terminal functional groups attached to aptamers is determined by the surface functionalization approach, such as linked to amine, thiol, or biotin termini (Odeh et al., 2020).

CHAPTER 3
METHODOLOGY

3.1 Research Framework

The following diagram outlines this research's methodological framework to prepare the SPR biosensor and for achieving the goals and objectives.



3.2 Experimental Section

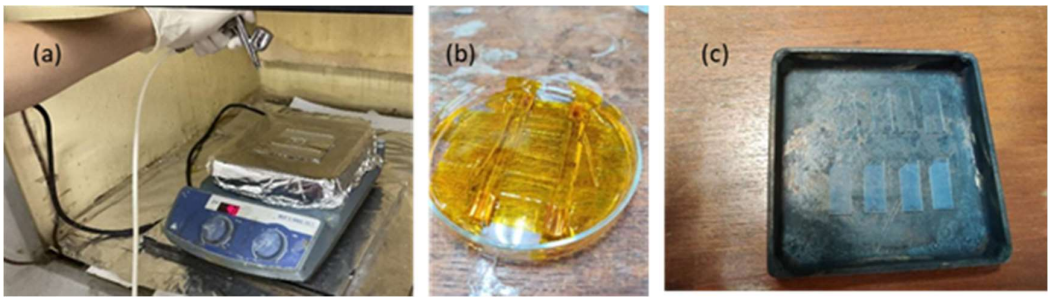
3.2.1 Synthesis of ZnO Nanorods on the Glass Surface

a. Preparation of ZnO seeds. The glass substrate is cleaned using acetone, ethanol, and DI water for 5 minutes with bath sonication. Next, the ZnO seed layer is prepared by dissolving Zinc acetate ($Zn(CH_3COO)_2$) in Ethanol (CH_3CH_2OH) at a concentration of 1 mM. To form the ZnO seeds, the prepared solution is sprayed on the glass surface, and placed on a hot plate with a temperature of 90 °C, at a distance of around 10 cm, to avoid the formation of droplets when the spray nozzle is too close to the sample (see the Figure 3.1 (a)). After coating, the sample is heated in the oven for 5 hours at 250 °C. After that, the sample is to be cooled spontaneously, enabling the formation of a white ZnO seeds coating.

b. Fabrication of ZnO nanorods. An aqueous solution of zinc nitrate ($Zn(NO_3)_2$, 10 mM, 20 mL) is mixed with an aqueous solution of hexamine ($C_6H_{12}N_4$, 10 mM, 20 mL) under bath sonication for 10 minutes. After that, turn the seed substrate that is already prepared for growing the nanorods upside down and put the sample on the step that prepares from the cutting glass slide to be like the ladder, which the solution can pass through, pour the prepared zinc nitrate and hexamine solution over the substrate, and heated for one hour on low power mode (400W) in the microwave (see the Figure 3.1 (b)). The procedure is repeated 5 times, after that the sample is rinsed with DI water and annealed in a furnace at 350 °C for 1 to form the ZnO nanorod layer on the glass substrate (see the Figure 3.1 (c)).

Figure 3.1

Synthesize of ZnO Nanorods



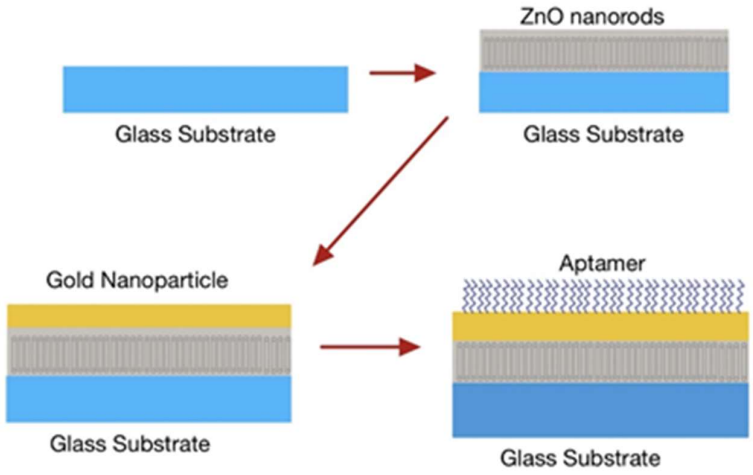
Note. (a) The ZnO first layer is made by spraying the ZnO seed on the glass surface. (b) To form the rods on the Seed layer, turn the ZnO seed substrate upside down and heat it in the microwave. (c) The sample is then annealed in the furnace to produce ZnO nanorods.

3.2.2 Synthesis of Gold Nanoparticles on ZnO by Utilizing Photoreduction Method

Gold nanoparticles (Au NPs) on the ZnO nanorods are produced following a straightforward photoreduction method, that requires only a UV source at a wavelength of 373 nm, to induce the formation of Au NPs (see Figure 3.2) (Chen et al., 2021; Courrol et al., 2007).

Figure 3.2

Fabrication of Biosensor



Note. Diagrams showing the proposed sensor's production processes of the SPR aptamer-based biosensor.

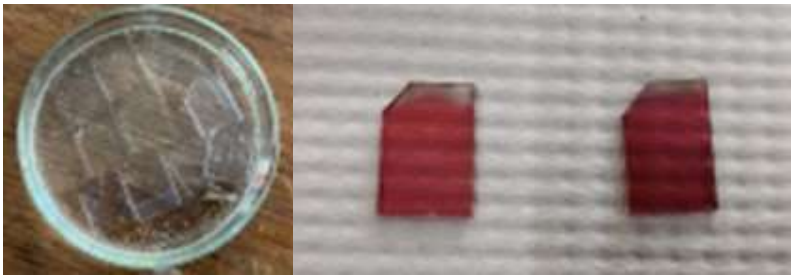
A solution of tetrachloroauric acid ($HAuCl_4$) at a concentration of 0.2 mM is prepared in a solvent mixture made of equal volumes of water and ethanol. The ZnO nanorods are immersed in 10 mL of such solution.

The Au (III) cations are subsequently reduced to Au (0) on the ZnO nanorods by irradiating at 373 nm for 10 minutes with UV light (6 W) (Eustis et al., 2005), followed by rinsing with DI water. The resulting Au size has a diameter of 40 nm. The procedure is repeated 3 times to increase the number and diameter of the Au NPs.

In the final step, the sample is annealed in the furnace for one hour at 450 °C, and let to cool naturally afterward (see Figure 3.3).

Figure 3.3

Results of Gold Deposition



Note. ZnO before and after deposit gold nano particle by irradiated with UV light for 10 minutes.

3.2.3 Surface Functionalization of Aptamer on the Glass Substrate

The Au/ZnO SPR surface must be functionalized with an aptamer for the specific detection of the analyte, in this case, glycated albumin. The aptamer employed in this study is provided with a thiol termination, which is the anchoring point for the Au/ZnO surface (Sasar et al., 2020). It is usually protected as a disulfide bridge with a short sacrificial molecule (C3) at the 3' end. The sequence is as follows:

5′-GGTGGCTGGAGGGGGCGCGAACGTTTTTTTTTT-3′–S-S-C3

The deposition of thiol-modified aptamer on the gold ZnO nanoparticle is done by first deprotecting the thiol group with TCEP (tris (2-carboxyethyl) phosphine), and then by immersing the sample in the aptamer solution at different concentrations.

a. Preparation of aptamer stock solution. According to the manufacturer, the aptamer must be diluted with 327 µl of TE buffer pH 8.0 which is composed of Tris and EDTA buffer (Tris serves as a nucleic acid solution, while the EDTA buffer prevents DNA damage), to obtain a stock solution with a concentration of 100 µM; therefore, several aliquots were prepared and frozen, ready to be thawed and used for the surface functionalization.

b. Thiol group deprotection. A 40 mM solution of TCEP is prepared by dissolving 0.08 g of TCEP with TE buffer 7 ml. TCEP solution is the end product, but it is an acidic solution of around 2.4 pH and cannot be used with aptamers (see Figure 3.4). Therefore, add NaOH 5.9 ml to increase the pH of the solution up to 7 pH. Then, DI water 1.1 ml was added to increase the volume of the solution up to 14 ml. as a result, the final product is TCEP 20 mM concentration in 14 ml of volume.

The obtained solution is stored at -20 °C. For deprotection, a 1:1: a mixture of TCEP and aptamer is incubated at room temperature for 1 hour, then diluted with TE buffer to the desired concentration. The Au/ZnO nanorods surface is immersed to allow for the binding, with different incubation times of 30, 60, and 90 minutes. The optimization is monitored by observing the peak shift of the aptamer *vs.* the incubation time, choosing by monitoring the absorbance peak that has reached the saturation time, or when the absorbance peak is slightly changing to no change at all.

Figure 3.4

Deprotecting the Thiol Group with TCEP

Note. TECP is added to the aptamer solution to break down disulfide bonds, and NaOH is added to adjust the pH.

3.3 Glycation of Human Serum Albumin with Fructose.

Albumin is transformed into glycated albumin, which is present in the form of proteins, through glycation, a non-enzymatic natural process in which a reducing sugar is bonded to a free amino group (Khoirunnisa et al., 2019). This outcome is also known as the Maillard reaction (Khoirunnisa et al., 2019). The typical glycation method involves the use of reducing sugars glucose and fructose (Sattarahmady et al., 2007), which form a covalent bonding with the protein (Kisugi et al., 2007). Glycation of albumin can be performed on both human serum albumin (HSA) and bovine serum albumin (BSA) because their structures are very similar (Arasteh et al., 2014). In this research, the preparation of glycated albumin has been performed on HSA. The procedure involves incubation with fructose for 7 days under magnetic stirring at 150 RPM (see Figure 3.5) (Sattarahmady et al., 2007). Human serum albumin is dissolved in 0.1 M phosphate-buffered saline (PBS) pH 7.4 at a concentration of 66 mg/ml, in presence of fructose at a concentration of 500 mM. After incubation, the glycated albumin in PBS is diluted to a concentration of 2 mg/ml. UV-Vis spectrophotometry and fluorescence spectroscopy are used to analyze the glycated albumin.

Figure 3.5

Incubation of Glycated Albumin

Note. Incubation of glycated albumin for 7 days by using albumin and fructose.

3.4 Characterization Methods of the LSPR Biosensor

Scanning electron microscopy (SEM) (JEOL model JSM-7800F (Prime)) is used to examine the morphology of ZnO nanorods and ZnO gold nanoparticle surfaces, at a resolution as low as 15 nanometers (Geetha et al., 2016).

UV-Vis Spectrophotometry (USB4000-UV-VIS) which measures from 200-850 nm is used to determine the light absorption of the Au/ZnO substrate in presence of aptamer and glycated albumin. HSA is analyzed before and after glycation. Furthermore, to determine specific vs. non-specific binding of the glycated albumin, a peak shift comparison is performed between the surfaces with and without the aptamer.

Glycated albumin is also analyzed using fluorescence spectroscopy (FluoroMax-3), as glycation affects ligand binding and causes structural changes in the albumin molecule. As a result, fluorescence spectroscopy can detect the glycation process, with high sensitivity, rapidity, and simplicity, making it an excellent tool for this investigation (Szkudlarek et al., 2017).

3.5 Detection of Glycated Albumin Testing the Absorbance Peak Shift

This experiment aims to examine the specificity of the aptamer binding with the glycated albumin, by measuring the UV-Vis spectrum of the system and comparing the sample before functionalization of the aptamer with the sample after adding glycated albumin. To measure this difference, the peak of gold, usually in the range of 520 nm to 550 nm, is monitored for any intensity or peak shift after adding glycated albumin.

To observe non-specific binding, the same experiment is repeated in absence of the aptamer, letting the glycated albumin interact directly with the whole Au-ZnO surface. By obtaining the difference between the aptamer-functionalized surface and the bare surface, it is possible to estimate the number of glycated albumins that bind with Au-ZnO nanorods.

3.6 Sensitivity of the Detection Glycated Albumin (Limit of detection, LOD)

To test the lowest concentration of an analyte on the sample surface that can be reliably identified, a.k.a. the limit of detection (LOD), several concentrations of glycated albumin are used to observe the peak shift: 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml, prepared in 0.1 M phosphate-buffered saline (PBS) pH 7.4. An aptamer concentration of 4 µM is used for the preparation of the substrates, which are immersed for 2 hours in a test tube containing 500 µl of glycated albumin. After that, the sample is removed and rinsed with TE buffer, dried, and measured at the UV-Vis spectrophotometer.

CHAPTER 4
RESULTS AND DISCUSSION

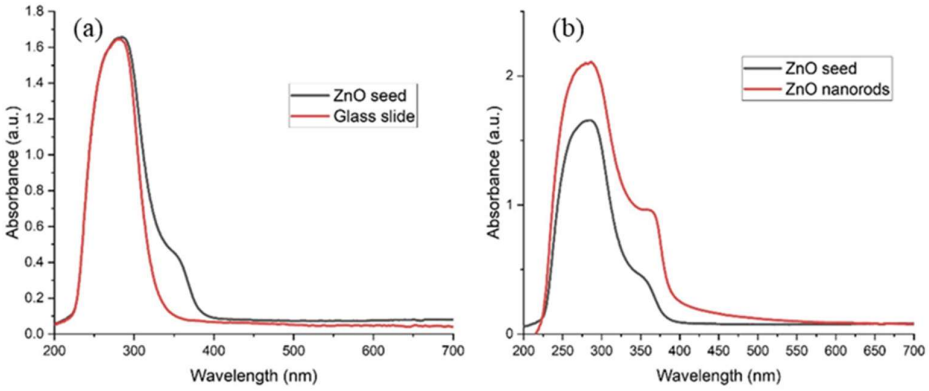
4.1 Synthesis of ZnO Nanorods

4.1.1 Characterization by UV Visible Spectroscopy

To form ZnO nanostructures by hydrothermal method, the starting procedure requires to initially deposit a thin ZnO seed layer on the substrate, which acts as a base for the ZnO nanorods. This ZnO seed film is characterized via UV visible spectroscopy, and the presence of a band around 350 nm is typical of the presence of the ZnO seed layer (Liu et al., 2006). The optical absorbance of ZnO seed grown on glass substrates as investigated as a function of wavelength in the 200-700 nm range is presented in Figure 4.1 (a). The result shows a shoulder at about 350 nm, confirming the presence of ZnO on the glass substrate, the latter having a broad absorption centered at about 280 nm.

Figure 4.1

UV Visible of Spectroscopy of ZnO



Note. (a) Characterization of UV visible spectroscopy of ZnO seed layer compared with a glass slide. (b) Characterization of UV visible spectroscopy of ZnO nanorods and ZnO seed.

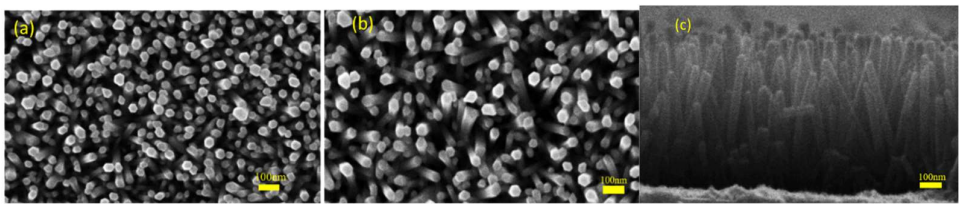
The subsequent growth of the ZnO nanorods from the hydrothermal growth method fabricated by heating the ZnO seed with the Zn nitrate and Hexamine for 1 hour in 5 cycles can also be confirmed by using UV visible spectroscopy. The peak related to ZnO, as shown in Figure 4.1 (b), is slightly red-shifted from 350 to ca. 360 nm, and it is more intense than the previous ZnO seed layer because the structure of ZnO was changed from thin film to the nanorods (Ridhuan et al., 2012).

4.1.2 Evaluate the Morphology of the Grown ZnO Nanorods Structure's Surface

Scanning Electron Microscopy (SEM) was used to examine the structure of the ZnO nanorods and determine their shape and size. Two concentrations of both Zn nitrate and hexamine at 5 mM and 10 mM were evaluated. After thermal treatment at 450 ºC in the furnace, the surface of ZnO with the 5 mM treatment (Figure 4.2 (b)) is composed of hexagonally shaped ZnO nanorods with a diameter of 43 ± 13 nm, whereas increasing the concentration to 10 mM the diameter increases to ca. 52 ± 8 nm (Figure 4.2 (a)). In both cases, the average length of the ZnO nanorods is about 600 nm (Figure 4.2 (c)). The nanostructured materials examinations reveal the linear trend between the concentration of the precursors and the resulting diameter of the nanorods. At lower concentrations, the diameter of ZnO is smaller than at higher concentrations, while increasing the concentration of the seed layer of ZnO during the formation stage induces the length of ZnO nanorods to increase (Ridhuan et al., 2012).

Figure 4.2

Morphologies of ZnO Nanorods



Note. The morphologies of the produced nanorods are shown in SEM images. (a) ZnO nanorods at 5 mM concentration (b) ZnO nanorods at 10 mM concentration. (c) The thickness of the ZnO nanorods.

4.2 Depositions of Gold Nanoparticles

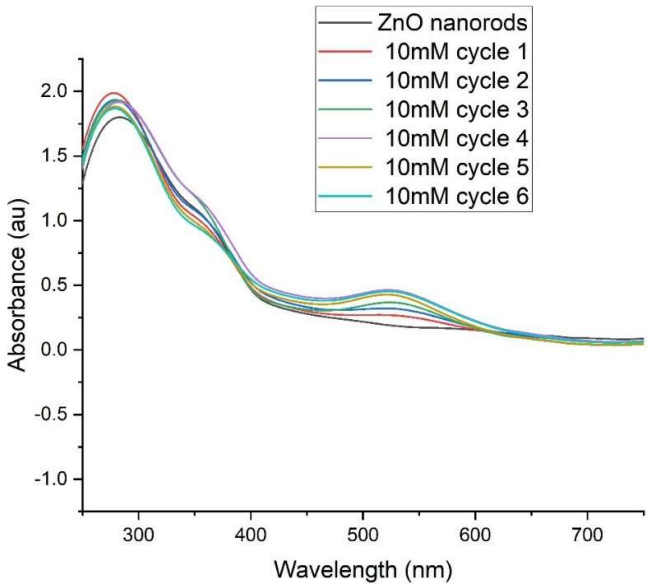
4.2.1 Ultraviolet-visible Spectroscopy (UV-Vis)

The deposition of gold nanoparticles on the ZnO nanorods can be monitored by UV visible spectroscopy, obtaining a plasmon resonance absorption range for gold particles between 510 and 550 nm depending on the size and concentration of ZnO. Figure 4.3 shows the absorbance of gold nanoparticles at various deposition cycles (up to 6), before and after deposition.

The significant peak at ca. 350 nm represents ZnO nanorods, while the increasing peak at around 520 nm indicates the formation of gold nanoparticles that aggregate on the ZnO nanorods (Li et al., 2019). The increasing intensity depends on the deposition cycle of gold.

Figure 4.3

Various Cycle of Gold Depositions



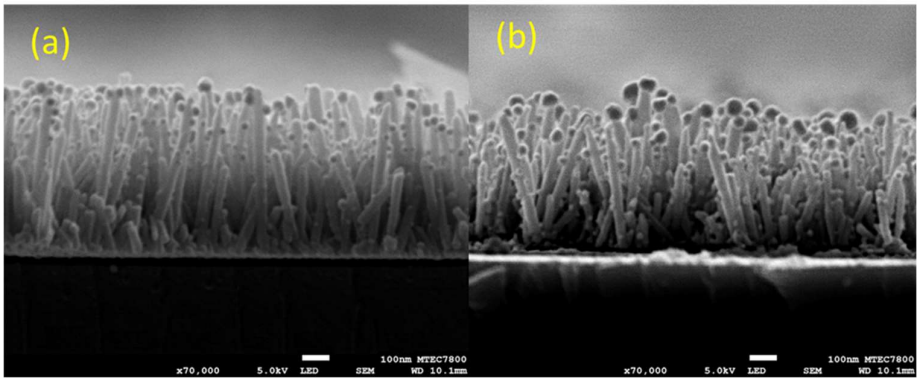
Note. Various cycles of gold deposition compared with ZnO nanorods without gold deposition were tested by measuring the absorption of gold using UV visible spectroscopy.

4.2.2 Scanning Electron Microscopy (SEM)

The SEM morphological characterization of the ZnO/Au systems obtained after 3 and 6 cycles is reported in Figure 4.4 (a) and 4.4 (b), respectively, as a side view. The reaction conditions used for both gold deposition processes are: 10 mM ZnO nanorod concentration and 10-minute UV exposure for each cycle. ZnO nanorods still retain a length of about 600 nm, while gold particles are represented by spherical shapes attached on top, and occasionally on the side of the nanorods. The size of the gold particles that accumulate on the ZnO nanorods changes over the period of gold deposition cycles, with a diameter variable between 40 nm (3 cycles) and 60 nm (6 cycles).

Figure 4.4

Morphologies of Gold on ZnO Nanorods



Note. Scanning electron microscopes of the deposition of gold on ZnO nanorods. (a) The three-cycle gold depositing method. (b) Gold is deposited with six cycles of deposition.

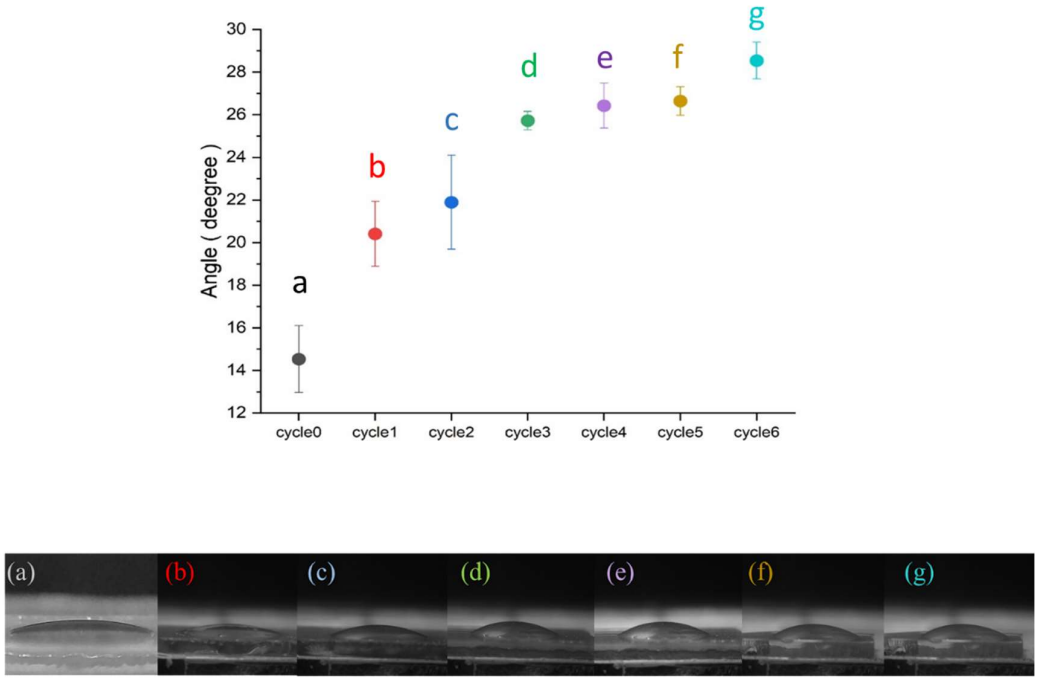
4.2.3 Water Contact Angle

The presence of hydroxyl groups on the surface of zinc oxide makes it spontaneously hydrophilic, with water contact angle (WCA) values between 15 and 30 degrees (Figure 4.5). When the Au is deposited the WCA increases with the increase of the number of deposition cycles. In details, the WCA increases as follows: 14.56º ± 3.00º (control),

18.99º ± 2.51 º (cycle 1), 20.35º ± 6.29 º (cycle 2), 25.73º ± 1.288 º (cycle 3), 26.05º ± 3.00 º (cycle 4), 26.64º ± 4.4 º (cycle 5), and 27.75º ± 5.24º (cycle 6). Therefore, upon Au deposition, the more the cycles the lower the surface hydrophilicity.

Figure 4.5

Surface's Water Contact Angle of Au-ZnO Nanorods



Note. The several cycles of gold deposition affect the surface's water contact angle which are (a) deposition of gold cycle 0, (b) deposition of gold cycle 1, (c) deposition of gold cycle 2, (d) deposition of gold cycle 3, (e) deposition of gold cycle 4, (f) deposition of gold cycle 5, (g) deposition of gold cycle 6.

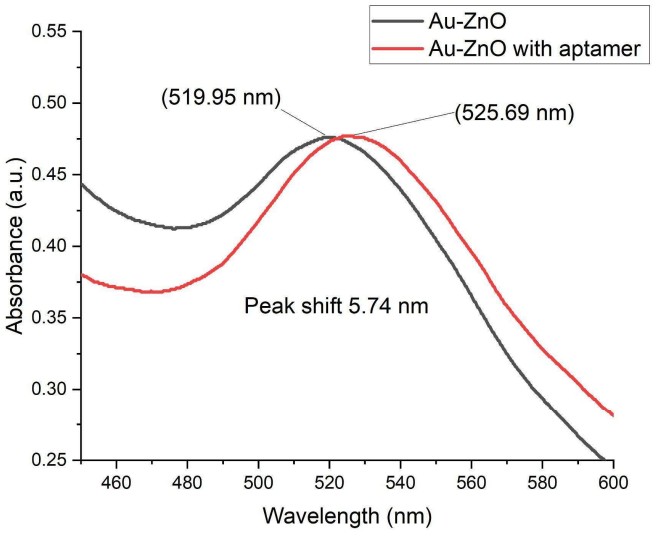
4.3 Functionalization of the Gold ZnO Nanorods with the Aptamer Solution

The functionalization of Au/ZnO with the aptamer specific for glycated albumin can be assessed via UV spectrophotometry, by monitoring the peak position of the Au

absorption (centered at 519 nm in Figure 4.6). Upon functionalization with the aptamer (200 uL, concentration: 4 µM), a Localized Surface Plasmon Resonance (LSPR) peak shift to 525 nm occurs because the aptamer binds to the gold, causing a change in the Au NP diameter, therefore inducing a change in the local refractive index, which is reflected in this red-shift (Nie et al., 2014), therefore demonstrating that the aptamer was successfully attached to the surface of the gold ZnO nanorods.

Figure 4.6

Functionalization of Aptamer



Note. The LSPR peak shifts at the spectrum of gold wavelength during functionalization with the aptamer.

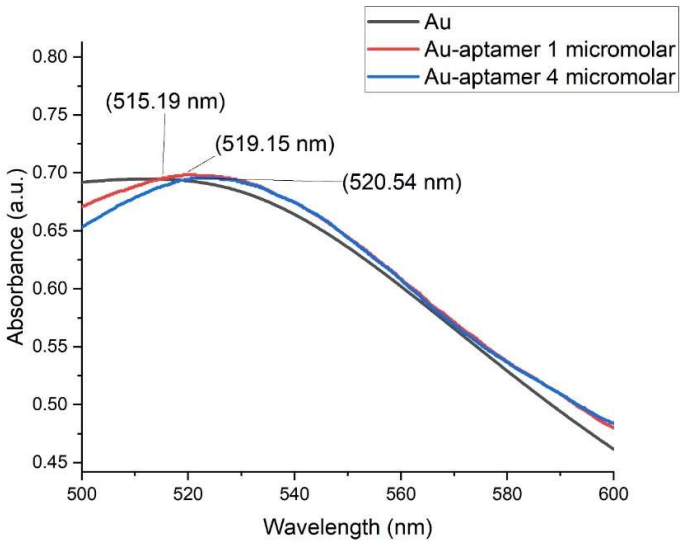
4.3.1 Aptamer Concentration

Aptamer concentrations 1 µM and 4 µM were chosen to optimize its functionalization on the Au nanoparticles. The reaction was conducted for 90 minutes at room temperature. The results reported in Figure 4.7 shows that at the lowest concentration (1 µM), the LSPR redshift is ca. 4 nm, whereas the highest concentration (4 µM) shows a slightly larger redshift of about 5.5 nm. Considering that the higher redshift is due to

more coating on the Au surface, the optimum concentration for the surface functionalization of the aptamer was set to 4 µM.

Figure 4.7

Concentration of Aptamer



Note. The peak shift of the 1 µM and 4 µM concentrations of aptamer was measured by UV visible spectroscopy.

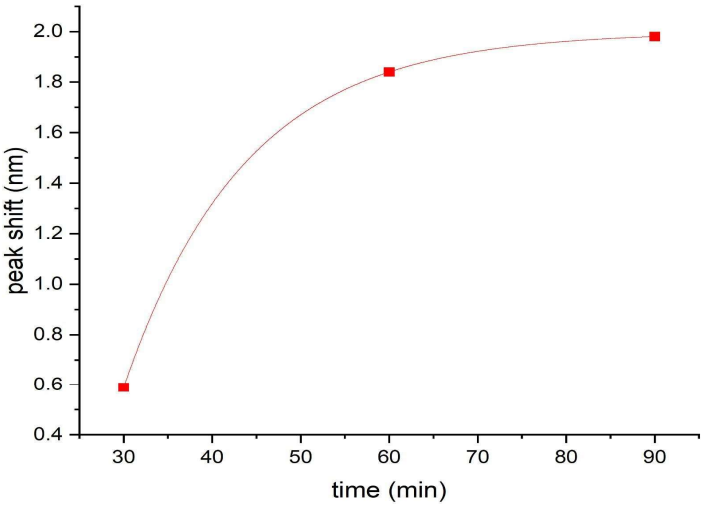
4.3.2 Incubation time of the Au/ZnO Nanorods with the Aptamer Solution.

A kinetic experiment was conducted at the aptamer concentration of 4 µM for 30, 60, and 90 minutes, to ascertain which concentration is the most appropriate for the surface functionalization of the Au/ZnO nanorods. The graph reported in Figure 4.8 indicates that the peak shift of absorbance after 30 minutes of incubation time is quite negligible, only ca. 0.6 nm, practically comparable to the instrumental error. After 60 minutes, a peak shift of around 1.8 nm can be identified, which increases to ca 1.9 nm at 90 minutes. Even though the variations in the LSPR peak shift are numerically limited, a trend in the incubation time can be observed. Although the increase at 90 minutes is small over the 60 minutes mark, most probably due to the surface being almost

completely saturated, this additional time can provide a further presence of aptamer and therefore increase the signal in the detection of glycated albumin. To get the highest coverage, the incubation time of the gold ZnO nanorods on the aptamer 4 µM concentration has been set at 90 minutes.

Figure 4.8

Incubation Time of Aptamer

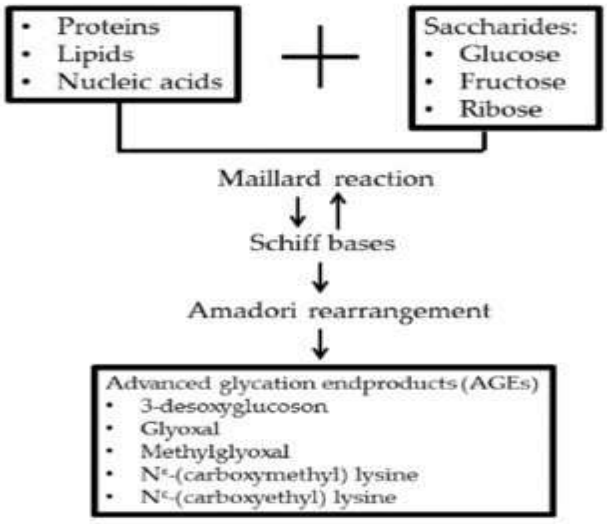


Note. Incubation time trend the Au/ZnO nanorods. The trend line is only for visual enhancement of the data points.

4.3.3 Preparation of Glycated Albumin

Figure 4.9

Maillard Reaction



Note. Maillard reaction produces a formation of advanced-glycation end-products (AGE). (Shen et al., 2020).

The complicated mechanism known as the Maillard reaction results in the formation of glycated albumin, as shown in Figure 4.9. In the initial stage of glycation, the non-enzymatic interaction between a reducing sugar (e.g., glucose, fructose, etc.) and the free amino groups of proteins produces the Amadori product, a glycated protein (Yoseph & Demo, 2015). The following step leads to the creation of advanced glycation end-products (AGEs) (Ahmadzadeh, 2014). The presence of these AGEs, which comprises tryptophan residue, can be detected using a fluorescence spectrophotometer (Sattarahmady et al., 2007). Therefore, the glycation of albumin is here monitored by using fluorescence spectrophotometry, using a slit width of 5 nm and a wavelengths λ_{exc} = 295 nm, the emission spectrum was recorded, using pure (non-glycated) albumin at day 0 incubation as the reference.

Figure 4.10 (a) reports the emission spectra of the albumin and the glycated albumin obtained at 7 days of reaction. The intensity of the emission in the first case is centered

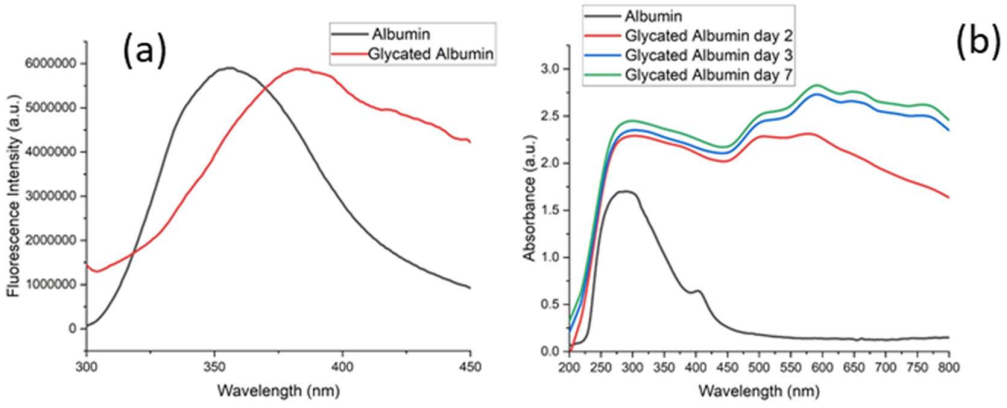
at around 350 nm, whereas the peak is shifted to about 375 nm after glycation. This phenomenon occurred due to the structural rearrangement surrounding tryptophan residue in the glycated albumin when compared to the residues in the native albumin (Szkudlarek et al., 2017).

The incubation of albumin was also monitored by using UV visible spectroscopy in the 200-800 nm wavelength lengths on days 2, 3, and 7. Clear changes occur during stirring at room temperature with fructose, as evidenced in Figure 4.10 (b). Already from day 2, the glycation process induces structural changes reflected by the increased intensity at 280 nm, which can be related to the conformational change around aromatic amino acids, and the appearance of multiple overlapping broad bands, especially in the 500-650 nm range, potentially due to the reaction of fructose with amino residual groups (Szkudlarek et al., 2017).

As a combined result from both UV and fluorescence analysis, albumin is predicted to have been successfully glycated after 7 days of incubation with fructose.

Figure 4.10

Results of Glycated Albumin



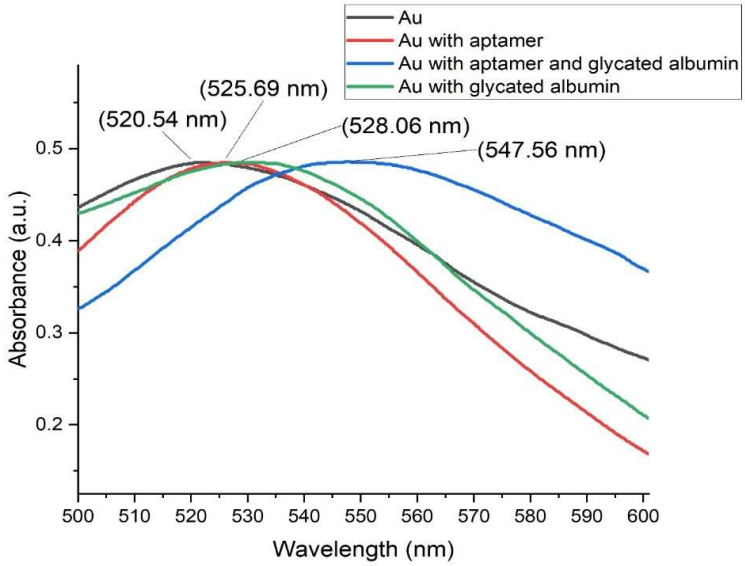
Note. a) Glycated albumin detection by using fluorescence spectrophotometer; b) UV visible spectroscopy.

4.3.4 Efficiency of the Aptamer Binding Towards Glycated Albumin

The Au/ZnO nanorods have been examined with and without the aptamer to determine both specific and non-specific binding of glycated albumin, and thus the sensor's efficiency. This is especially relevant in the case of albumin, which has a great affinity toward many hydrophilic substrates (Muslimov et al., 2021). The interaction between gold ZnO nanorods (in the absence or presence of the aptamer) and the glycated albumin is assessed by measuring the absorbance before and after the addition of 200 µl of albumin solution, in PBS buffer at a concentration of 2 mg/ml, on the gold ZnO nanorods surface (Figure 4.11) , for the non-functionalized sample, a limited redshift from ca. 520 to 528 nm (8 nm of peak shift), whereas the aptamer-functionalized shows a wider redshift to 547 nm (27 nm of peak shift). The results indicate that the non-specific binding is quite limited as it does not produce appreciable redshift on the gold nanoparticles, whereas the three-fold increase in the peak-shift observed for the sample with aptamer functionalization evidence a significant enhancement of the binding efficiency of glycated albumin.

Figure 4.11

Peak Shift of UV Visible Spectroscopy



Note. The peak shift of UV visible spectroscopy with aptamer and without aptamer.

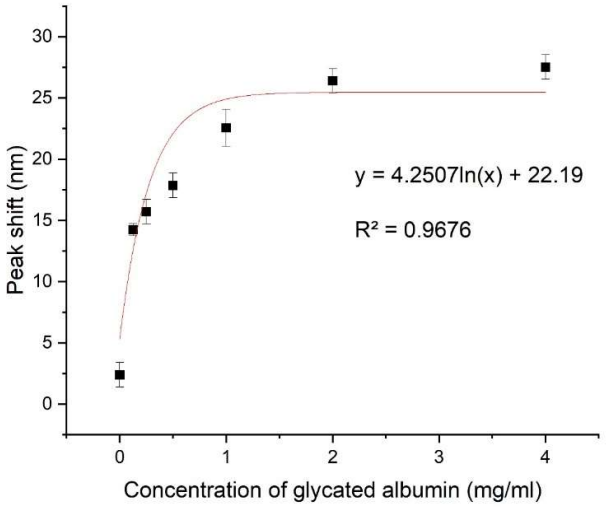
4.4 Sensitivity and Selectivity Test of the Aptamer with Glycated Albumin.

4.4.1 Sensitivity Test

To have an appreciation of the sensitivity of the aptamer binding to various concentrations of glycated albumin, samples were exposed to a range of concentrations of glycated albumin between 0 and 4 mg/mL, measuring the absorbance peak shift intensity of gold nanoparticles in the range of 200-900 nm via UV visible spectrophotometry. According to Figure 4.12, the peak shift increases with an almost exponential trend along the series. It is noteworthy that peak shift data below the 0.125 mg/ml concentration of glycated albumin might not be sufficiently reliable due to its very low concentration measured via UV visible spectroscopy. Beyond 2 mg/mL, the limited peak shift is justifiable by saturation of the aptamer already at this concentration. The aptamer may not be able to attach to an excess of glycated albumin, resulting in a negligible change in size or refractive index and therefore in the absorbance peak shift. As a result, there was an exponential fitting in the trend with an error bar depicting the standard deviations of measurements gathered from three experiments. Equation $y = 4.2507\ln(x) + 22.19$, $R^2 = 0.9676$ was calculated using the relation of the concentration of glycated albumin and the LSPR peak shift. The limit of detection of aptamer functionalization on the gold ZnO nanorods interacting with glycated albumin was estimated to have the lowest concentration of a measurement that can effectively measure is 1.408 mg/ml.

Figure 4.12

Sensitivity of the Aptamer



Note. Limits of detection of the aptamer functionalized on gold ZnO nanorods interact with glycated albumin in various concentrations.

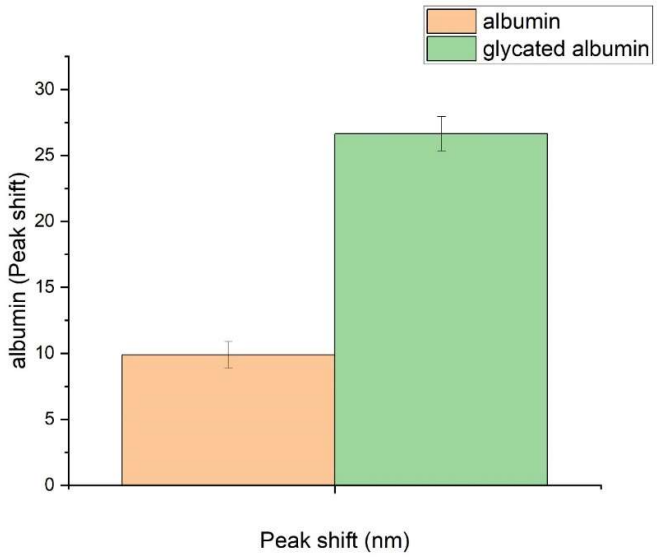
4.4.2 Selectivity Testing

To assess the selectivity of the aptamer sensor towards the analyte, a binding experiment in presence of either standard serum albumin or glycated albumin was performed, maintaining the same conditions in both cases, that is 2 mg/ml of protein, and samples were prepared using an aptamer concentration of 4 μM. The comparison of the LSPR peak shifts I reported in Figure 4.13. While the normal albumin shows a peak shift of about 9 nm, comparable to a similar peak shift previously obtained in absence of aptamer (see Figure 4.13), only the correct analyte, represented by the glycated albumin, shows a much higher peak shift of about 27 nm, this result demonstrates the significant difference between using very similar substrates, that are pure serum albumin vs. glycated albumin, as analytes for aptamer binding. Since this aptamer has been optimized to specifically bind glycated albumin rather than pure albumin (Sasar et al., 2020), this characteristic can be used to confirm the selectivity of

the aptamer. This behavior can be useful once applied to the analysis of glycated albumin even in presence of normal serum albumin.

Figure 4.13

Selectivity of the Aptamer



Note. The results of testing the aptamer specific binding with both pure albumin and glycated albumin.

CHAPTER 5
CONCLUSION

5.1 Conclusion

In summary, the main goal of this research is to design and fabricate a simple biosensor that surfaces formed of gold nanoparticles which were synthesized on ZnO using the photoreduction process to improve signal and recognize a signal from a metallic surface. Hydrothermal growth processes are being used to produce ZnO nanorods. ZnO was coated on the glass slide to be used as a base for the sensor. The Characterization of SEM was performed to determine the morphology of hexagonal ZnO nanorods at 5 mM concentration which has a diameter of 43 ± 13 nm, whereas the diameter of 10 mM is 52 ± 8 nm.

Such a biomarker Glycated albumin concentration was successfully quantified using DNA aptamers as specific recognition elements for glycated albumin. The aptamers sequence 5′ -GGTGGCTGGAGGGGGCGCGAACGTTTTTTTTTT-3′ – S-S-C3 was used to bind with the glycated albumin to enhance the output signal in terms of SPR peak shift. The ability of aptamer to bind to glycated albumin was tested at 2 concentrations, 1 μM and 4 μM. The result showed that 4 μM gives a higher peak shift compared to 1 μM concentration. The incubation time of aptamers binding with gold nanoparticles was tested at 30, 60, and 90 minutes, and the result showed that the absorbance peak reach the saturation time in 90 minutes at room temperature. When these conditions are met, the aptamer has a suitable condition to bind with glycated albumin.

To prepare the glycated albumin, the human serum albumin was used to incubate with fructose for seven days at room temperature in the magnetic stirring. The fluorescent spectroscopy and UV visible spectroscopy were used to confirm the glycated albumin by observing the change of absorbance with UV vis and change in the intensity of the emission with a fluorescence spectrophotometer.

To determine the success of the detection, UV Visible Spectroscopy was used to analyze the peak shift of the aptamers when binding to glycated albumin. The result demonstrates that peak shift is related to glycated albumin concentration which higher concentration occurred higher peak shift. To evaluate the detection performance of the system, Gold ZnO with aptamers and without aptamers were performed to analyze the nonspecific binding of glycated albumin with gold ZnO substrate which the result showed the enhancement of the signal compared to the substrate without aptamer.

The signal output-sensitive response in only glycated albumin and other protein are not affected by the detection. Moreover, the experiment was performed to test the sensitivity of the detection of glycated albumin the lowest concentration that can be detected is 1.408 mg/ml according to the LOD testing.

5.2 Recommendation and Future Implications

Various research has been proving that biosensors can be more deeply explored in numerous ways to assist clinical fields in processing an early detection of life-threatening diseases. The application of numerous scientific fields of the detection method improves the detection's accuracy and precision, allowing for immediate treatment and the potential to save more lives. According to this research glycated albumin has been implemented as a biomarker, and it has shown a great result that can be applied to real-world infectious diseases. Therefore, in the future, it could be assumable that if this research has been taken further in experimenting with antigens, it could possibly lead to another open door in clinical and medical research, another step forward in modern diagnosis methods.

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