DESIGN AND FABRICATION OF IMMUNOCHROMATOGRAPHIC STRIP BIOSENSOR FOR BREAST CANCER PROTEIN BIOMARKERS ANALYSIS

BY

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عمادة الدراسات العليا
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Dedication

To

My beloved parents

My dear sisters and brothers

My faithful friends

ACKNOWLEDGMENTS

First and foremost I offer my sincerest gratitude to Allah, the most gracious and the most merciful, who gave me the strength to achieve this work and guide my way in the life.

I would like to express my very great appreciation to my advisor, Dr. Abdel-Nasser Kawde, for his valuable and constructive support, patient guidance, and enthusiastic encouragement during the research work. His willingness to give his time so generously has been very much appreciated. Also I would like to express my deep gratitude to my committee members, Professor Abdullah Abulkibash and Dr. Tawfik Saleh for providing me with their valuable comments and feedback to improve my work.

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LIST OF ABBREVIATIONS

IgG	:	Immunoglobulin G antigen
CEA	:	Carcinoembryonic antigen
EIA	:	Enzyme immunoassay
RIA	:	Radioimmunoassay
FIA	:	Fluorescent immunoassay
ELISA	:	Enzyme linked immunosorbent assay
IFA	:	Immunofluorescence
DFA	:	Fluorescent antibody test
Incr E	:	Potential increment
AuNPs	:	The gold nanoparticles
BSA	:	Bovine serum albumen

ABSTRACT

Full Name : Wael Mhd Amen Mahfoz

Thesis Title : Design and Fabrication of Immunochromatographic Strip Biosensor for Breast Cancer Protein Biomarkers Analysis

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The current research work explores the design and fabrication of two types of immunosensor magnetic beads based electrochemical immunosensor and lateral-flow chromatographic strip that can be used for various sensing and analytical applications. To fabricate the magnetic beads based electrochemical immunosensor, the rabbit IgG antigen was used as a model. After preparation of the streptavidin-coated magnetic beads solution, we used biotinylated anti-rabbit IgG followed with alkaline phosphatase conjugated anti-rabbit IgG to form the sandwich compound and produce α–naphthol after adding α-naphthyl phosphate substrate. To detect the product of α–naphthol and transduce the biosensor signal, electrochemical analysis is selected using both of cyclic and square wave voltammetry techniques for the qualitative and quantitative detections. Graphite pencil electrode as a working electrode characterized with many advantages like high selectivity, renewable surface, low cost and simplicity of use is chosen to achieve a high signal for α–naphthol electrooxidation. The optimization study is done in both sides of biosensor design and electrochemical detection. As a result, the detection was achieved successfully with a relative standard deviation below 4%. Besides, the sensor allows the limit of quantification of 1 ng/ml and the detection limit (at 3σ) was estimated to be 0.16 ng/ml. The biosensor as recorded in this work achieves the detection target with a remarkable outcome and distinct future potential enhancement.

The second part of research is the design and fabrication of a low-cost, simple, easy-to-use without complex and expensive instrumentation, and disposable dry biosensor (lateral-flow chromatographic strip) that can be used for rabbit IgG detection. The work was achieved on many steps, starting with synthesize of AuNPs (size of 15 n.m) followed by the fabrication process of gold nanoparticles-based dry immunosensor includes pretreatment of membranes and applied biojet chemical system, batch laminating system and the guillotine cutting system. For quantitative detection, the strip portable reader used to record the signal. To develop the performance of the lateral flow produced strip, the optimization was achieved over many components, membranes and solutions used in the preparation. The detection was achieved successfully with a relative standard deviation equals 4.4%. Besides, the sensor allows the limit of quantification of 1 ng/ml and the limit of detection (at 3σ) was estimated to be 0.48 ng/ml. The features of the fabricated biosensors make such biosensors ideal for applications in breast cancer early diagnosis, rapid screening, point-of-care (POC) detection in hospital laboratories, and even for near-patient or home testing.

ملخص الرسالة

الاسم الكامل: وائل محمد أمين محفوظ

عنوان الرسالة: تصميم وتصنيع الحساس البيولوجي المناعي الكرموتغرافي لتحليل البروتينات المرتبطة بسرطان الثدي

التخصص: الكيمياء

تاريخ الدرجة العلمية: ديسمبر ٢٠١٣

هذه الرسالة تعرض تصميم وتصنيع نوعين من الحساسات المناعية الحيوية وهما الحساس المناعي الكهروكيميائي ذو الجسيمات المغناطيسية و الحساس الكرموتغرافيا المناعي وكلا الحساسين يمكن استخدامها بتطبيقات تحليلية متنوعة. لتصميم الحساس المناعي الكهروكيميائي ذو الجسيمات المغناطيسية تم اختيار مولد الضد الغلُوبولين المَناعِيّ نمط ج (غ م ج) مُولد بمصل دم أرنب كهدف. لتحضير الحساس البيولوجي تم تحضير محلول الجسيمات المغناطسية المطلية بالستربتدوفيدين واسُتخدام غلوبولين مناعي نمط ج مرتبط بالبيوتين إضافة لغلوبولين المناعي نمط ج مرتبط بإنزيم الفوسفتيز القلوي لتشكيل مركب السندويش المناعي وإنتاج ألفا نفثول بعد إضافة ألفا نفثيل الفوسفات. لتحديد ألفا نفثول قد تم استخدام تقنية الفولتومتري الدائري و الفولتومتري الموجة المربعة لتحديد الكمي والكيفي. أما بالنسبة للقطب فقد تم استخدام قطب الغرافيت الرصاصي الذي يتميز بخصائص متعددة مثل الحساسية وإمكانية تجديد السطح وانخفاض التكلفة وسهولة الاستخدام. قد أجريت دراسة الشروط المثلى للحساس للشروط الحيوية و الشروط الكهروكيميائية وكنتيجة تم بنجاح تصنيع الحساس الحيوي وتحديد المادة الهدف بمستوى تحليل كمي ١ نانوغرام \ مل ومستوى تعيين 0.16 نانوغرام \ مل ودرجة تكرار التحليل بانحراف معياري نسبي أقل من ٤ %. وبهذا فإن تصميم الحساس البيولوجي الكهركيميائي قد تم بنجاح إضافة لإمكانيات ملحوظة لتطوير الحساس البيولوجي وتحسين أداه.

الجزء الثاني من البحث يدور حول إنتاج حساس بيولوجي من نمط شرائح الكروموتغرافيا المناعية كأداة سهلة وقليلة التكلفة وسهلة الاستعمال للتحليل. الحساس المصمم يمكن استخدامه لتحليل مولد الضد الغلُوبولين المَناعِيّ نمط ج (غ م ج) مُولد بمصل دم أرنب. تصميم الحساس تم على عدة مراحل بداية من تصنيع جسيمات نانو لمعدن الذهب (بقياس ١٥ نانومتر) ثم تم تصميم شريحة الحساس بمعالجة الأغشية المستخدمة وتطبيق الرش للمواد الحيوية على الأغشية متبوعة بتجميع الأغشية وتقطيع الشريحة الناتجة إلى شرائح متساوية بالحجم والعرض. لتطوير أداء الحساس فقد تم دراسة الشروط المثلى للمحاليل والأغشية المستخدمة. والحساس المصمم قد نجح في تحليل المادة الهدف لمولد الضد الغلُوبولين المَناعِيّ نمط ج (غ م ج) مُولد بمصل دم أرنب بمستوى تحليل كمي ١ نانوغرام \مل ومستوى تعيين ٠,٤٨ نانوغرام \مل ودرجة تكرار التحليل بانحراف معياري نسبي بقيمة ٤,٤ %. ميزات الحساس البيولوجي المصمم تمكن من استخدامه في العديد من التطبيقات مثل تشخيص المبكر لسرطان الثدي إضافة لسرعة التحليل وإمكانية استخدامه بالمخابر والمستشفيات وحتى في المنازل بشكل شخصي.

CHAPTER 1

Introduction

High speed analysis, simplicity of using, high selectivity, ability of industrial and medical applied and sensitivity of the target analytes detection are some advantages provided by biosensor science. Since 1962, when first biosensor application was offered by made by Professor Leland Clark in the form of an amperometric enzyme electrode for glucose detection, the biosensor became as a one of the most active research fields and provided the society and medicine with a lot of benefits in the pathological diagnosis and detection of different types of analytes in the chemistry, water analysis, food and agriculture fields. Under developing and fabrication in the world laboratories, biosensor science represents a magnificent and promising area in the advanced analytical chemistry and a benefit technology for the future of human.

1.1 Definition

A biosensor is a device for the detection of different kind of analytes which comprises a biological recognition element and a suitable transducer, which are coupled to an appropriate data processing system (Figure 1.1).

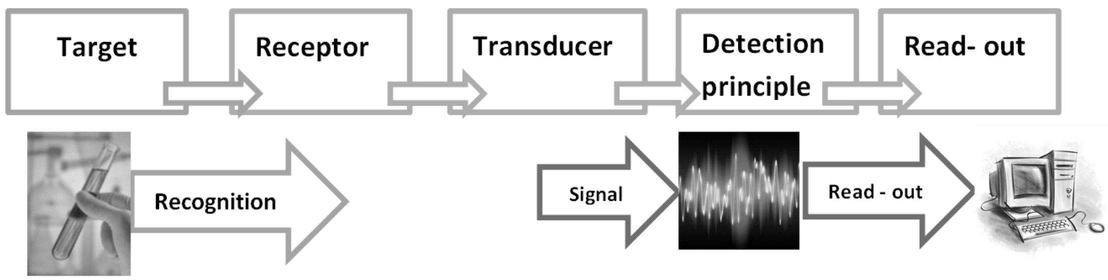

Figure 1.1: A scheme represents the biosensor.

1.2 Components of biosensor

In general, biosensor consists of three parts, which are bioreceptors or molecular recognizers, Signal transducers or detector elements and associated electronics or signal processors (Figure 1.2).

1- Bioreceptors (or molecular recognizers): this part contains sensitive biological elements which can be reacted selectivity with the targets. The biological recognition element may be an enzyme, micro-organism, tissue or bioligand such as antibodies and nucleic acids and they can be created by biological engineering or extracted from natural resources.
2- Signal transducers or detector elements: transforms the signal resulting from the interaction of the analyte with the biological element into another signal that can be more easily measured and quantified. The transducers work in a physicochemical way, optical, piezoelectric, and electrochemical and depend on the changing of pH of solution, heat, light, mass or electrons transferring to transform the signal. Many types of transducers used in the biosensor including electrodes, semiconducting pH electrodes, thermistors, photo detectors and piezoelectric medium.
3- Associated electronics or signal processors: Those are primarily responsible for the display of the results in a user-friendly way and include all of amplifiers, filters, multiplexers, analog-to-digital, converters, linearizers and compressors.

A common example of a commercial biosensor is the blood glucose biosensor, which uses the enzyme glucose oxidase to break blood glucose down (Figure 1.3). In doing so it first oxidizes glucose and uses two electrons to reduce the FAD (a component of the enzyme) to $FADH_2$. This in turn is oxidized by the electrode (accepting two electrons from the electrode) in a number of steps. The resulting current is a measure of the concentration of glucose. In this case, the electrode is the transducer and the enzyme is the biologically active component.

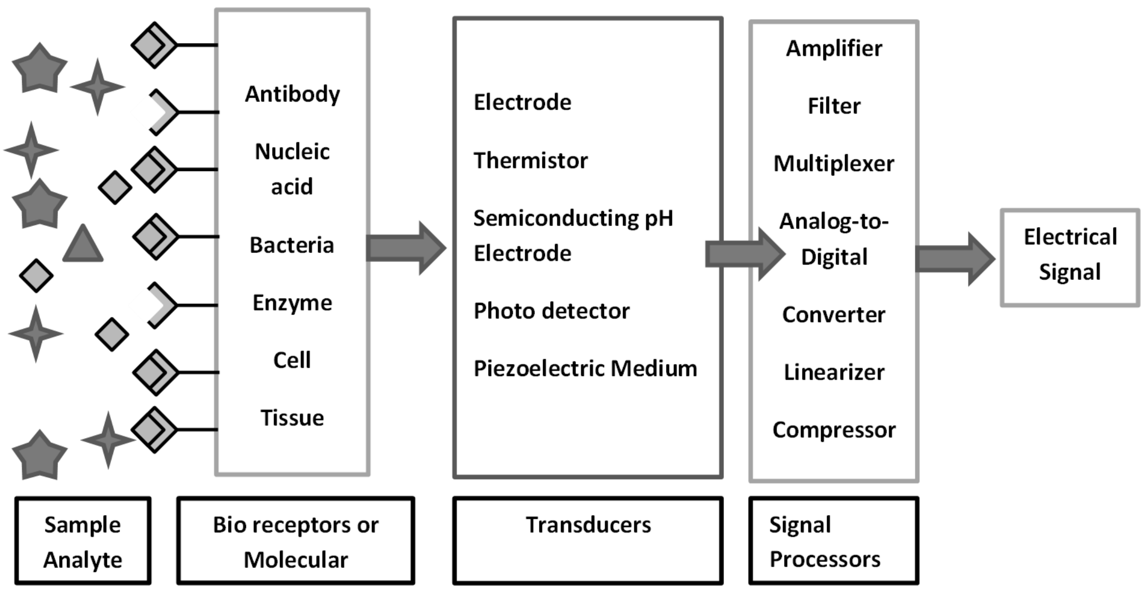

Figure 1.2: A scheme represents the components of biosensor.

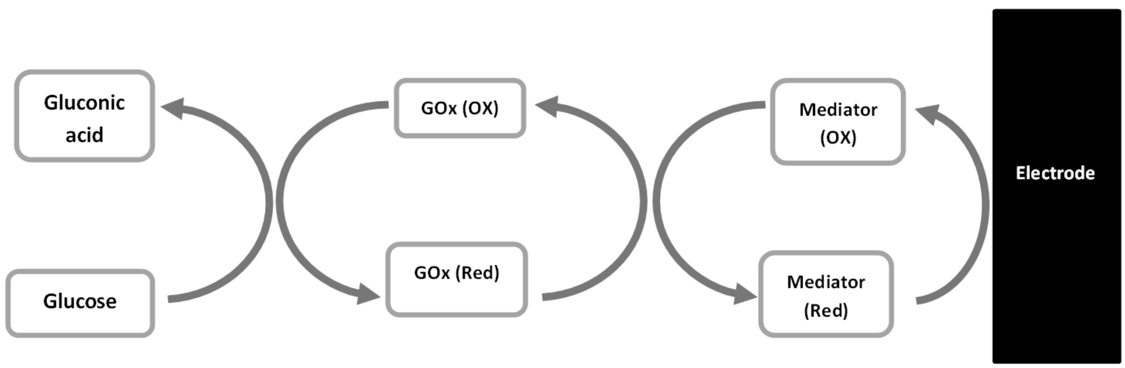

Figure 1.3: A scheme represents a glucose biosensor.

1.3 History of biosensors

In 1956, Professor Leland C Clack published a paper about oxygen electrode as an introduction in the biosensor science. Discovery of antibody structure was done between 1959 and 1962 which will be an important compound in the developing process of immuno-biosenor. In 1960, Radioimmunoassay was first described by Rosalyn Sussman Yalow and Solomon Berson.

Then in 1962, first described for biosensor was done by Clark and Lyon who described an amperometric enzyme electrode for glucose [1]. In 1966, a technique to prepare something like immunosorbent to fix antibody or antigen to the surface of a container was published. In 1971, Peter Perlmann and Eva Engvall at Stockholm University invented ELISA which will be one of the most wide common method used in the immunoassay.

In 1974, thermal transducers such as thermal enzyme probes and enzyme thermistors were proposed. The idea of Clark came to reality in1975, when glucose analyser lunched by Yellow Springs Instrument Company, the idea of sensor was based on the amperometric detection of hydrogen peroxide and become as a first biosensor laboratory model used for commercial purposes [2]. In the same year, the idea of bacteria could be harnessed as the biological element in microbial electrodes for the measurement of alcohol suggested. Furthermore, Lubbers and Opitz coined the term optode, which was a fiber-optic sensor with immobilized indicator to measure carbon dioxide or oxygen. The concept was used to develop the optical biosensor for alcohol detection. In addition of that, Generation of the first monoclonal antibodies was done by George Kohler and Cesar Milstein which will contribute in the developing of immunoassay.

In 1976 electrochemical glucose biosensor, in a bedside artificial pancreas, was marketed with the name of Biostator. On other side, La Roche introduced the Lactate Analyser LA 640 which was used to shuttle electrons from lactate dehydrogenase to an electrode. This is an important forerunner for lactate analyzers for sports and clinical application. In 1982, the first needle-type enzyme electrode for subcutaneous implantation was reported in vivo application of glucose biosensors. Followed in 1984, a paper on the use of ferrocene and its derivatives as an immobilized mediator for use with oxidoreductases was published. In 1987 a pen style glucose sensor was launched by MediSense Company, which has subsequently become one of the most popular glucose biosensor [3]. In 1996, the statistics showed that the sale of the home blood glucose monitoring reached 175 million dollars. In 2001, Several other glucose biosensors have been developed and more than 40 different brands are available on the market [4].

1.4 Classification of biosensors

Biosensors are classified according to the recognition elements or the transducer used (Figure 1.4).

1.4.1 Biosensors classified according to the recognition element

Biosensors are classified according to the recognition element as bioaffinity sensors, enzyme sensors, DNA and RNA sensors, whole cell biosensors; or according to the transducer as electrochemical, electric, optical, piezoelectric and calorimetric sensors [5].

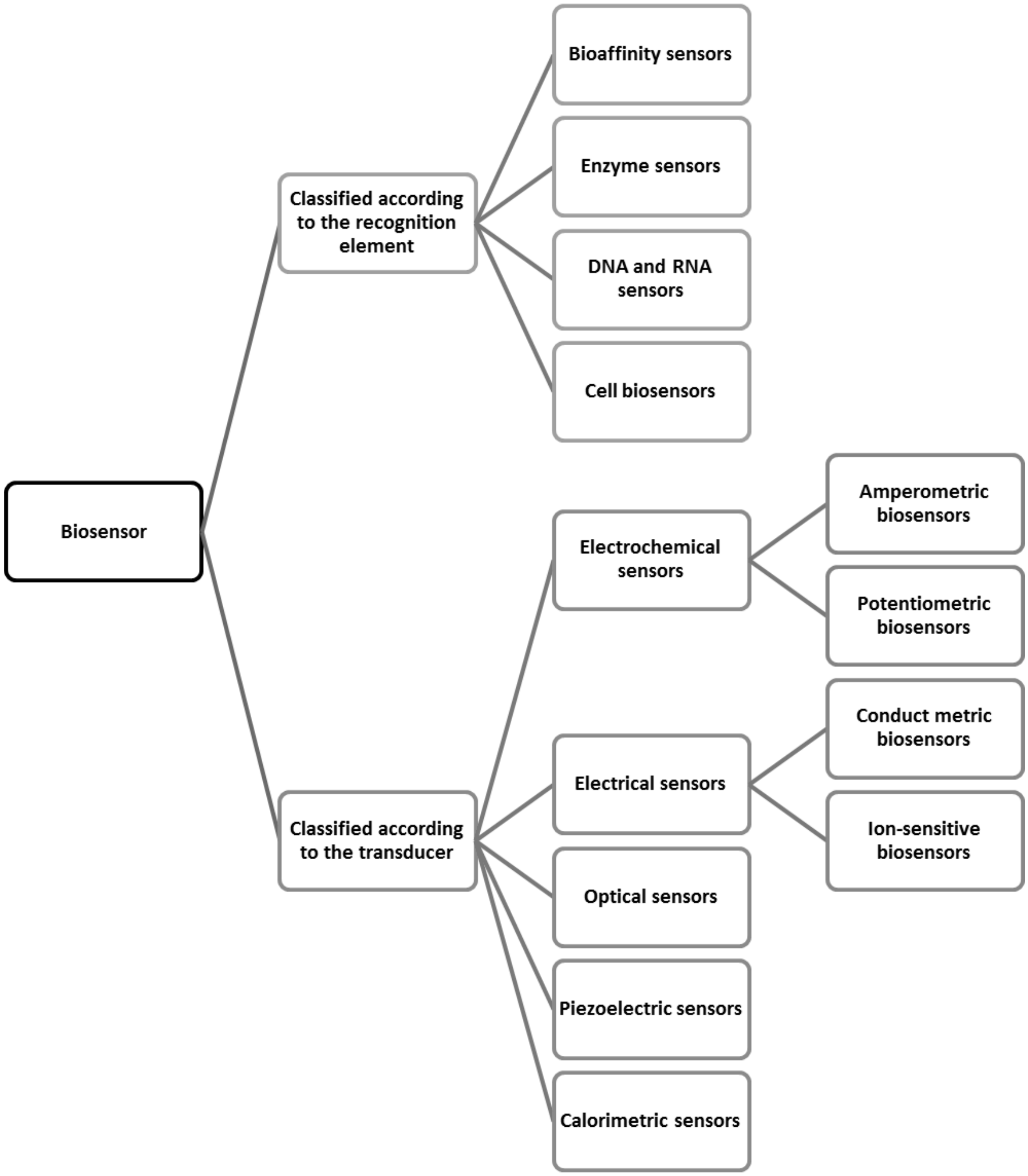

Figure 1.4: A scheme represents the classification of biosensors.

Bioaffinity sensors

A bioaffinity sensor uses immobilized hormone receptors or antibodies to detect hormones or antigens. Different immobilization techniques used to produce the bioaffinity sensors, these methods depend generally on one of the different chemical and physical bonding principles like physical adsorption, covalent binding, matrix entrapment, cross-linking and encapsulation.

Enzyme sensors

The enzyme biosensors utilize enzymes which are specific for the desired molecules and catalyze generation of the product, which is determined by using a transducer. For example, the biosensors used for measuring glucose in blood samples use glucose oxidase or glucose dehydrogenase enzymes.

DNA and RNA sensors

In these kinds of biosensors, the target analytes are generally different length of segments of DNA or RNA chain captured by the known sequence of bases in DNA molecule.

The complementary sequence, called a probe, which can be synthesized and subsequently labeled with an optically or electrochemically detectable compound like using a fluorescent label (Figure 1.5). Because the type of the target detected, these kinds of biosensors are considered as active research area to diagnosis different kinds of diseases like cancer.

Cell biosensors

These bioreceptors are either based on biorecognition by an entire cell (microorganism) or a specific cellular component that is capable of specific binding to certain species (Figure 1.6).

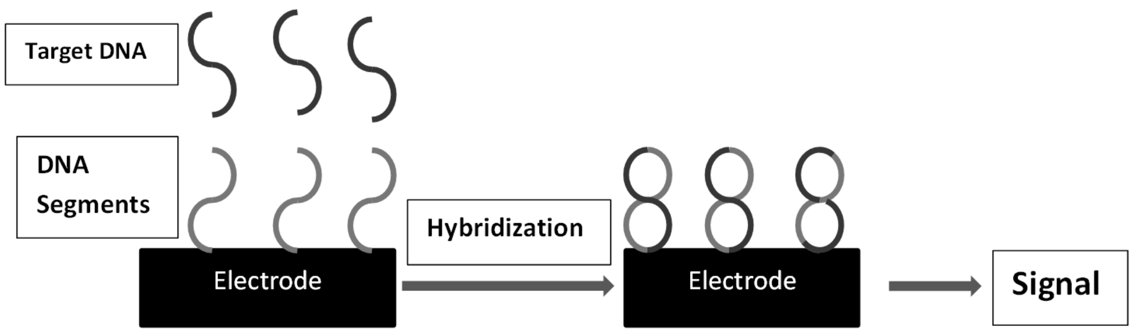

Figure 1.5: A scheme represents a DNA biosensor.

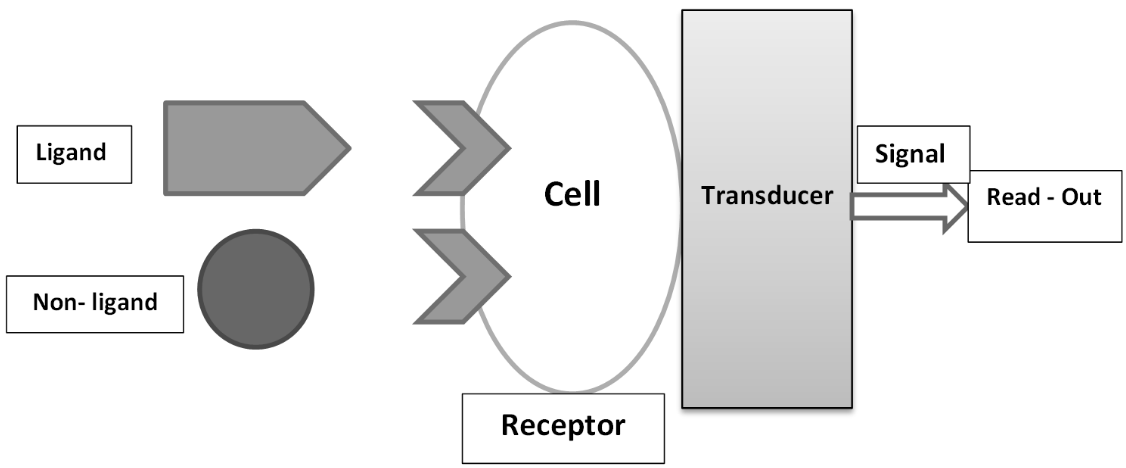

Figure 1.6: A scheme represents a cell-based biosensor.

1.4.2 Biosensors classified according to the transducer

Electrochemical sensors

In general, electrochemical sensors consist of Amperometric and Potentiometric biosensors.

1. Amperometric biosensors: As a definition amperometry is one of a family of electrochemical methods in which the potential applied to a sensing electrode is controlled instrumentally and the current occurring as a consequence of oxidation/reduction at the electrode surface is recorded as the analytical signal. In its simplest form, the applied potential is stepped to and then held at a constant value; and resulting current is measured as a function of time.

However, amperometric biosensor works by the production of a current when a potential is applied between two electrodes. They generally have response times, dynamic ranges and sensitivities.

The biochemicals can be detected and quantified amperometrically by their enzyme-catalyzed electro-oxidation or electroreduction, or their enzyme-catalyzed hydrolysis/phosphorylation followed by electro-oxidation/electroreduction or their involvement in a bioaffinity reaction enabling electro-oxidation/electroreduction. Amperometric biosensors are considered as the most wide spread class of biosensors.

2. Potentiometric biosensors: In the situation of the potentiometric biosensors, the transducer measures difference in potential that is generated across an ion-selective membrane separating two solutions at virtually zero current flow. Glass electrodes, metal oxide based sensors and ion-selective electrodes, are wide spread and common potentiometric transducers used in this type.

Electrical sensors

There are two types of electrical sensors conduct metric and ion-sensitive biosensors.

1. Conductmetric sensors: This type of sensors depends on measuring the electrical conductance or resistance of the solution which is changing due to produce of ions or electrons during the course of biochemical reaction.

2. Ion-sensitive biosensors: These Biosensors are based on ion-selective field-effect transistors and consist of a field-effect transistor with a gate formed by a separated reference electrode and attached to the gate area via an aqueous solution. The field-effect transistor has an ionsensitive surface. The surface electrical potential changes due to the interaction between ions and the semiconductor. This change in the potential can be subsequently measured. The transducer can be constructed by covering the sensor electrode with a selectively permeable polymer layer, through which ions may diffuse and cause a change in the field-effect transistor surface potential.

Optical sensors

The optical biosensor based on determining changes in light absorption between the reactants and products of reaction, or fluorescence when the device detects the change in frequency of electromagnetic radiation emission which is caused by previous absorption of radiation and also by generation of an excited state lasting for a very short time.

Piezoelectric sensors

This sensor depends on immobilization of biomolecules on the sensitive plate which is connected with piezoelectric component [6]. Usually the piezoelectric component consists from a quartz-crystal like quartz, tourmaline, lithium niobate or tantalate, oriented zinc oxide or aluminium nitrides that are sensitive for the piezoelectric effect and coated with gold electrodes.

Calorimetric or thermometric sensors

These biosensors are constructed by immobilization of biomolecules onto temperature sensors. Once the analyte comes in contact with the biocomponent, the reaction heat which is proportional to the analyte concentration is measured. The total heat produced or absorbed is proportional to the molar enthalpy and the total number of molecules in the reaction. The temperature sensor measures of changing in the heat and convert it into a signal which can be seen as a read-out.

CHAPTER 2

Magnetic beads-based electrochemical immunosensor

2.1 Introduction

Immunoassays are chemical tests used to detect or quantify a specific substance, the analyte, in a sample using an immunological reaction. The analytes include drugs, hormones, specific proteins and tumor markers in different kinds of sample like urine, serum blood, body liquids, food, water and environment samples. Immunoassays may be qualitative, positive or negative, by recording presence or absence of the signal or quantitative by measuring the tense of the electric, optical or chemical products signal produced by the immunological reaction.

2.1.1 Classification of immunoassay tests

1. Immunoprecipitation: The simplest immunoassay method measures the quantity of precipitate, which forms after the reagent antibody has incubated with the sample and reacted with its respective antigen to form an insoluble aggregate.
2. Particle immunoassays: By linking several antibodies to the particle, the particle is able to bind many antigen molecules simultaneously.
3. Immunonephelometry: The immediate union of antibody and antigen forms immune complexes that are too small to precipitate. However, these complexes will scatter incident light and can be measured using an instrument called a nephelometer.

4. Radioimmunoassay (RIA): is a method employing radioactive isotopes to label either the antigen or antibody. This isotope emits gamma rays, which are usually measured following removal of unbound free radiolabel.
5. Fluorescent immunoassay (FIA): refers to immunoassays which utilize a fluorescent label or an enzyme label which acts on the substrate to form a fluorescent product.
6. Enzyme immunoassay (EIA): is a method uses an enzyme to label either the antibody or antigen.

2.1.2 Enzyme immunoassay (EIA)

The sensitivity of EIA approaches that for RIA, without the danger posed by radioactive isotopes in addition of being easy to use and applies make from EIA as one of the most common techniques in the immunosensor filed. The enzymatic reaction produces the chemical product which can be determine optical or detect by using different electrochemical techniques.

2.1.2.1 Enzyme linked immunosorbent assay (ELISA)

ELISA test is one of the most popular immunoassay formats available for routine research and diagnostic use. It is increasingly being applied in clinical medicine for detecting proteins associated with disease. ELISA test uses a multi-well plate to apply multi immunoreactions and detect different kinds of antigen. The main principle of ELISA test is the synthesis of sandwich ELISA, this synthesis is done by a special procedure. The steps start with preparing the surface of the cells in the plate to which a known quantity of capture antibody is bound, then the nonspecific binding sites on the surface should be blocked by using special chemicals. The sample apply to the plate after the preparation, after that the plate is washed to remove the unbound antigen. Following that, a specific antibody is added and the sandwich compound is formed by stocking the antigen between the two antibodies. After the forming of the sandwich compound, Enzyme-linked secondary antibodies are applied and bind specifically to the antibody from the sandwich compound, the plate is washed again to remove the unbound antibody-enzyme conjugates. A chemical is applied to be reacted on the surface of the enzyme and converted into a color, fluorescent or electrochemical signal. In the last step the absorbency or fluorescence or electrochemical signal of the plate wells is measured to determine the presence and quantity of antigen. ELISA may be run for both qualitative and quantitative format. Qualitative results show the presence of absence the antigen in the sample. In quantitative test, the optical density of the sample is compared to a standard curve and shows the concentration of the antigen in the sample.

2.1.2.2 Magnetic beads based electrochemical immunosensor

Separation of the sandwich compound from the excess of the specific antibody labeled is considered as a very critical step in the immunoassay and the success the test depends in the first place on the success of the separation step. Many strategies are followed to succeed the separation progress. Using the magnetic beads allows for us a guaranteed method to achieve the separation in addition of presenting of a surface area to form the sandwich compound (Figure 2.1). Generally streptavidin magnetic beads to capture biotinylated molecules, and as a first step in the test the biotinylated anti-body is added into the streptavidin magnetic beads solution vessel, the biotinylated anti-body captured on the surface of the magnetic beads then after the apply the sample and in the case of presence of the antigen, it binds with the biotinylated anti-body on the beads surface. The specific label anti-body is added to form the sandwich compound by binding with the antigen and stacking on the magnetic beads surface. The rapid and reproducible isolation can be done by applying the magnetic field which attracted the beads. A chemical is applied to be reacted on the surface of the label anti-body and converted into a color, fluorescent or product which can be detected electrochemically.

In the current work, Magnetic beads based electrochemical immunoassay was applied to detect rabbit-IgG antigen (Figure 2.2). The streptavidin-coated magnetic beads are reacted with biotinylated anti-rabbit IgG. Then a sample contained rabbit IgG target can be added, followed with alkaline phosphatase conjugated-anti rabbit IgG adding to form the sandwich compound. α-Naphthyl phosphate disodium is used as a substrate and reacts on the phosphatase enzyme surface and produce α–naphthol as a product which can be detected using electrochemical transducer.

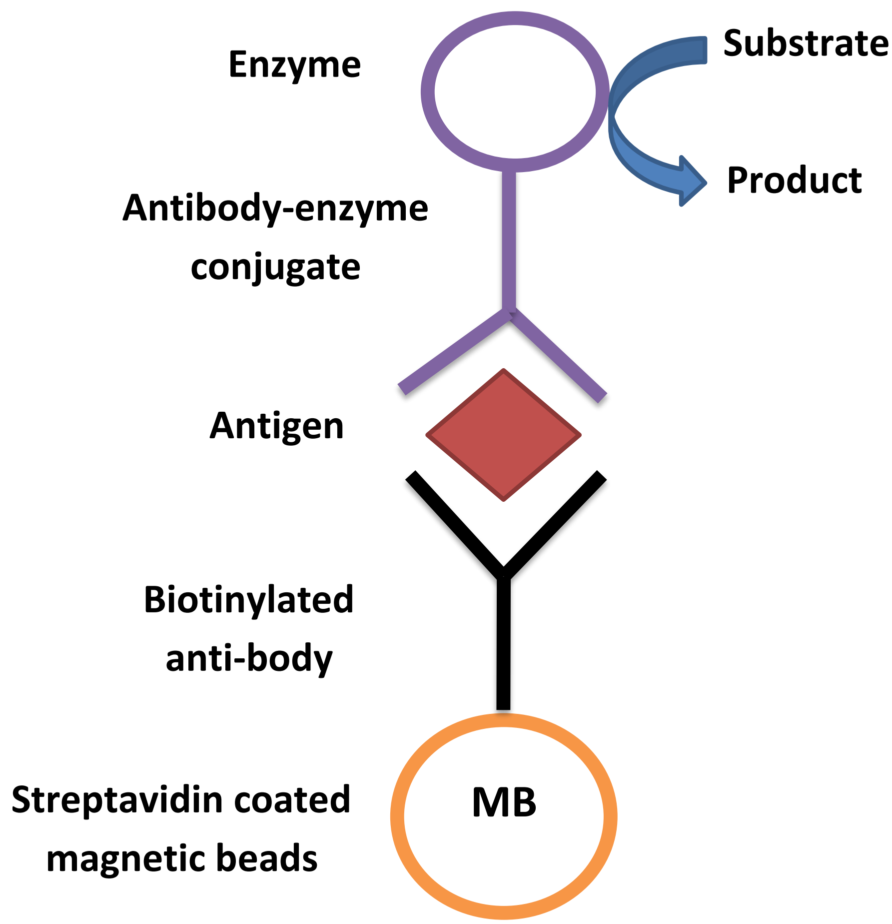

Figure 2.1: A schematic representation for the MB-antibody-antigen-antibody enzyme conjugate.

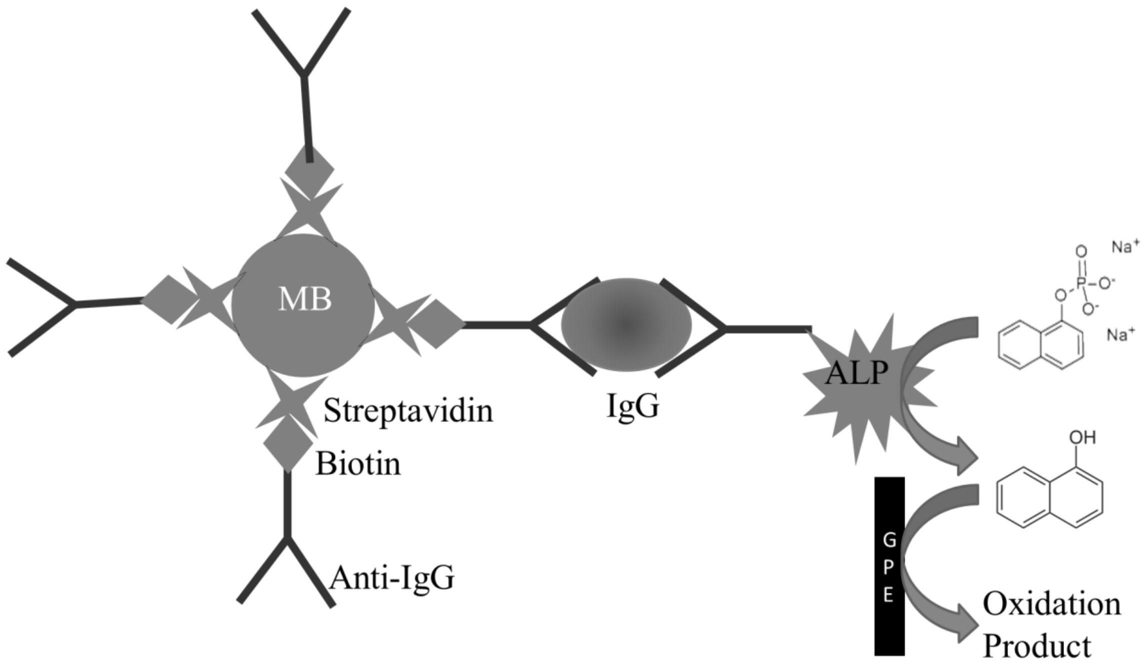

Figure 2.2: A schematic representation for the MB-antibody-antigen-antibody alkaline phosphate conjugate, followed by the enzymatic reaction of α-naphthyl phosphate disodium to α-naphthol and its electrochemical detection at the graphite pencil electrode (GPE).

2.1.2.3 Transducers used in immunoassay

The most common transducers used in immunoassay are colorimetric and electrochemical transducers; however other types of transducers can be applied in immunoassay. Here, colorimetric and electrochemical transducers will be concentrated for their importance as it is mentioned.

2.1.2.3.1 Colorimetric transducers

Optical immunoassays depends on many optical techniques includes fluorescence, surface plasmon resonance, absorbance and reflectance. In generally optical immunoassay is based on the interaction of antigen-antibody complex on inert surfaces. Specific binding of antibody increases the thickness of the reactants on the surface, and changes the color of the light reflected from the surface [7]. Furthermore, absorbance and transmittance can be used on a wide range in the case of producing a color product from the substrate in the enzyme immunoassay as they applied in ELISA.

In immunofluorescence (IFA), specific monoclonal or polyclonal antibodies are conjugated to fluorescent dyes (fluorochromes), which can be visualized using a fluorescence microscope, fluorometer, fluorescence scanner, or flow cytometer. Fluorophores are excited by light at a specific wavelength, and they release the extra energy by emitting light at another, longer wavelength. In a direct fluorescent antibody test (DFA) used for histochemistry the antigen-specific labeled antibody is applied to a fixed specimen on a microscope slide, incubated, washed, and visualized under a fluorescence microscope.

2.1.2.3.2 Electrochemical transducers

As mentioned in the chapter one and according to classification, electrochemical transducers consist of amperometric and potentiometric transducers. On the other hand, the applied methods of electrochemical transducers vary according to the kind of the recognition element used and the kind of the product which is formed from the enzymatic reaction. In the case of using the immobilization, the plate used for immobilized use as an electrode in the same time, therefore the plate is connected with other reference and counter electrodes and instruments to process the produced signal. Another method can be applied depending on the separate and extract the product produced from the enzymatic reaction and treat it as a separated sample. Here, the properties of the product compound are studied and different electrochemical technique in addition of the suitable working electrodes surface can be applied to achieve the qualitative and quantitative determination.

In our research, graphite pencil electrode used as a transducer to detect α–naphthol produced from the enzymatic reaction. To convert the electric signal, the cyclic voltammetry and square wave voltammetry techniques were applied in our work.

2.1.2.4 Electrochemical detection

The current research, the electrochemical techniques were used to complete the detection process of rabbit IgG using one type of carbon electrodes as working electrode so in this section some electrochemical principles and carbon electrodes information are explained to clarify the part linked with detection of α–naphthol.

2.1.2.4.1 Electroanalytical techniques

Electroanalytical chemistry is based on the study an analyte by measuring the potential and the current in an electrochemical cell containing the sample. In general, the electrochemical techniques are classified in two types including potentiometry or static techniques and dynamic techniques. In the static techniques no current is allowed to flow through the solution during the potential measurement of the solution. On the other hand, in dynamic techniques the current is allowed to flow through the solution for the measurements of both current and potential. In its turn, the dynamic techniques is divided into controlled current technique and controlled potential techniques which also is divided into fixed potential and variable potential techniques or what is called voltammetry’s techniques.

2.1.2.4.1.1 Voltammetry

Voltammetry is based on measurement of the cell's current while actively altering the cell's potential. In other words, Voltammetry is the study of current as a function of applied potential and curves produced voltammograms. Increasing the ratio between faradaic and non-faradaic current by lowering the background current helps to improve the detection limit of concentrations in the voltammetric techniques. However, there are many types of voltammetric techniques which differ from each other by varying of excitation waveform and current sampling regime.

I. Cyclic voltammetry

Cyclic voltammetry is the most widely used technique for qualitative electroanalysis. The method consists of scanning linearly the potential of a stationary working electrode using a triangular potential waveform (Figure 2.3). The recorded plot of current versus potential is termed a cyclic voltammogram and time is dependent function of a large number of physical and chemical parameters. To explain the cyclic voltammetry (Figure 2.4) represents a common case for cyclic voltammogram assumed that the oxidized form is present initially and the negative potential scan is chosen for the first half cycle. As the applied potential approaches the characteristic potential for the redox process, a cathodic current begins to increase and the figure hit a peak. The molecules formed in the half cycle and accumulated near the surface, are reoxidized to oxidized material and the anodic current increases and hit a peak.

II. Square wave voltammetry

In this method, a square wave is superimposed on the potential staircase sweep. Oxidation or reduction of species is registered as a peak or trough in the current signal at the potential at which the species begins to be oxidized or reduced (Figure 2.5).

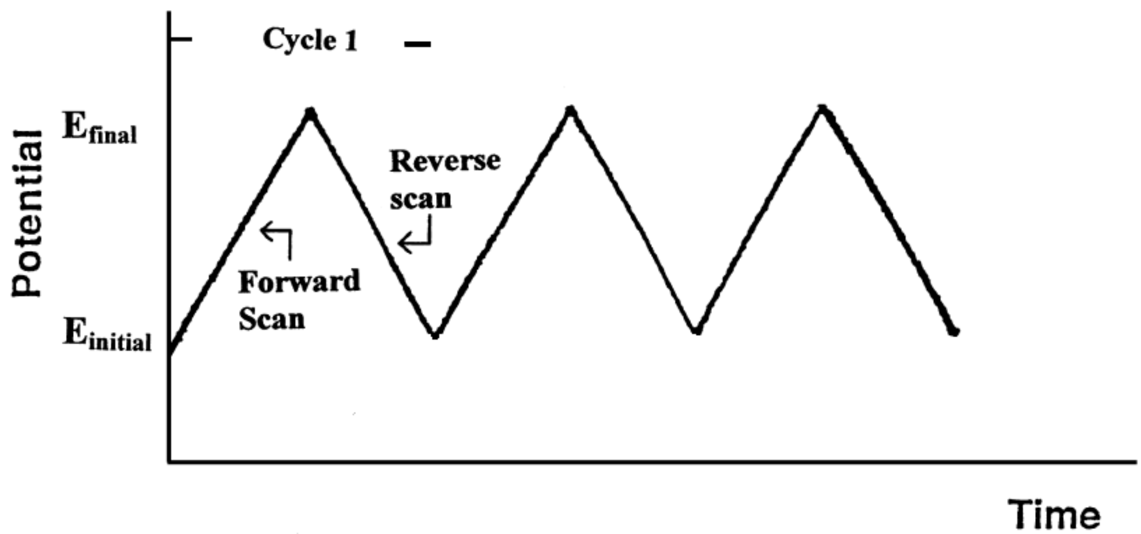

Figure 2.3: Potential time excitation signals in cyclic voltammetric experiment.

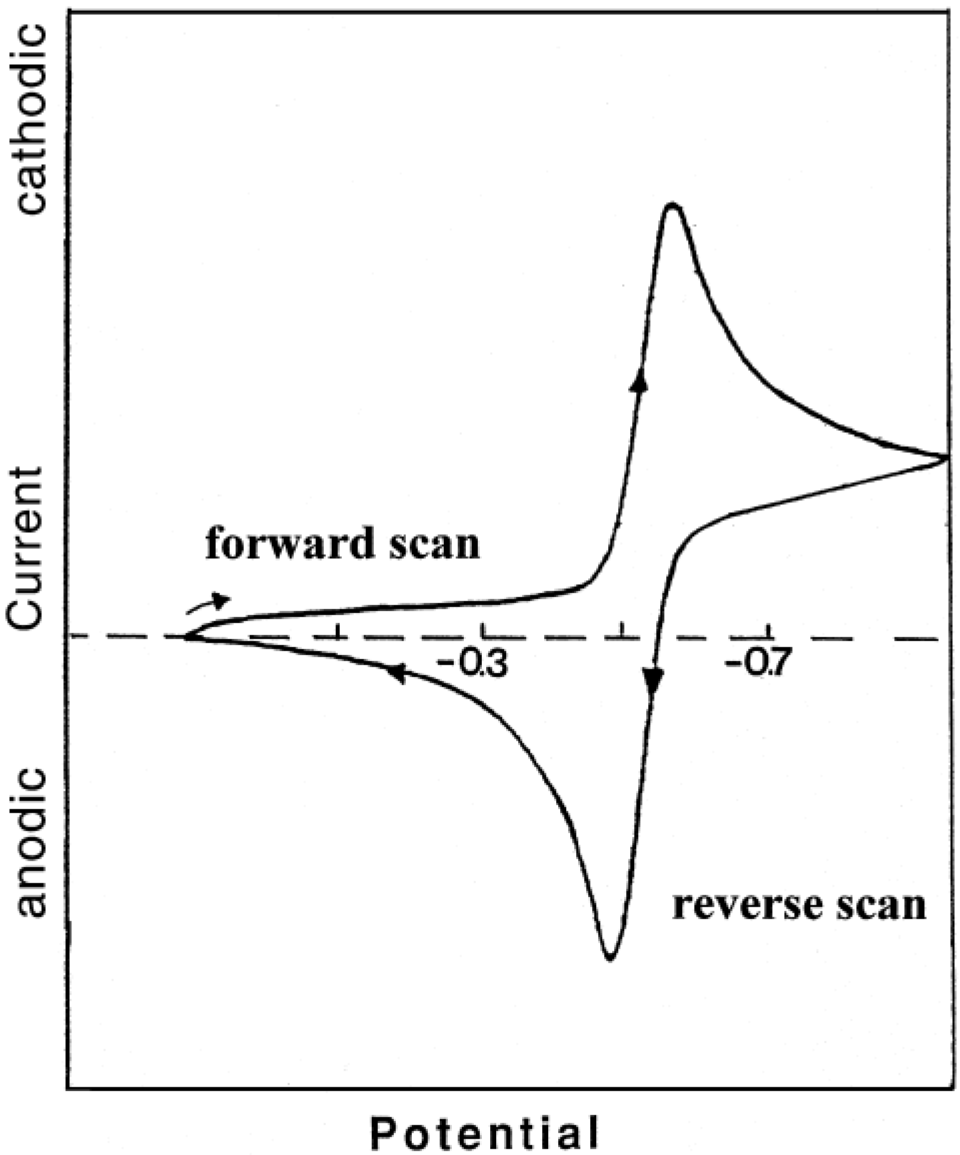

Figure 2.4: A typical cyclic voltammogram for a reversible redox reaction.

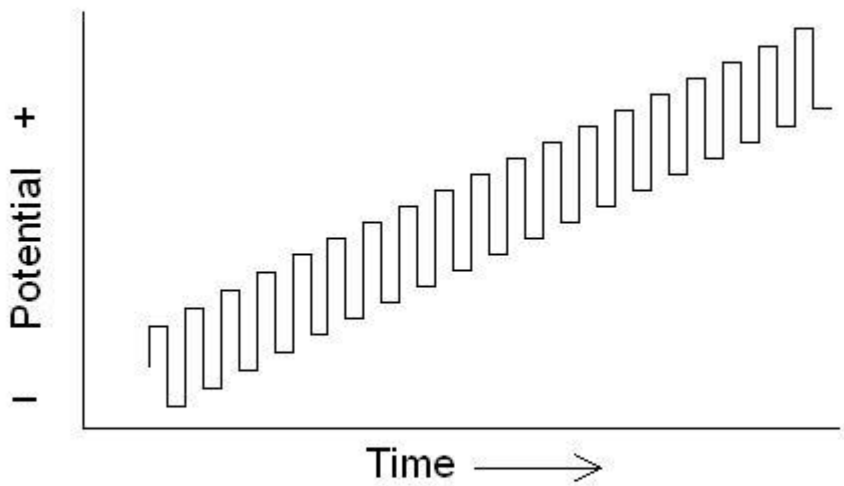

Figure 2.5: An excitation signal for square wave voltammetry.

2.1.2.4.2 Electroanalytical cell used in voltammetry

Voltammetry techniques use the three electrode system which contains reference, counter and working electrodes. In this system, the current is measured between the counter and working electrodes while the potential is measured between the reference and working electrodes.

2.1.2.4.2.1 Carbon electrodes

The carbon electrodes are used widely in the electrochemical analysis field. Furthermore, Characterizations like easy fabrication, cheap cost of manufacturing, and highly selectivity make the carbon electrodes required working electrodes in the three electrodes cell system. There are many types and designs of the carbon electrodes based on the carbon material used in the fabrication and the way of producing.

I. Glassy carbon electrode

The electrode is made from a non-graphitizing carbon which combines glassy and ceramic properties with those of graphite.

II. Graphite carbon paste electrode

The electrode is made from a mixture of conducting graphite powder and a pasting liquid like mineral oil. These electrodes are simple to make and offer an easily renewable surface for electron exchange. For its structure is considered as heterogeneous carbon electrodes.

III. Glassy carbon paste electrode

The electrode is made from a mixture of glassy carbon powder and a pasting liquid like mineral oil. In general, it has the same graphite carbon paste electrode properties in addition of those characterized the glassy carbon material.

IV. Graphite pencil electrode

In addition of being a common writing device, the graphite leads are used as a carbon electrode. In general, the graphite pencil electrode is used as a working electrode successfully, and provides many advantages during the measurement experiment like the renewable surface, ability of control the length of the leads used in the test and the low cost.

2.2 Literature review

Starting with first description as an amperometric enzyme electrode for glucose made by professor Leland C Clark Jnr, biosensor has given the possibility to determine different kinds of analytes like sugars [8, 9], nucleic acids basis [10, 11], oligonucleotides [12], ssDNA and RNA [13, 14], Diabetes [15, 16], and detection of basic sites of nucleic acids [17]. Immunoassay is one of the most wide biosensor applications and includes different kind of techniques.

According to its importance for different social sections like medicine, food and water analysis, and during the last three decades the researches have been focusing on the developing of immunoassay systems. Magnetic beads based immunoassay provides a huge development for the simplicity of the immunosensor test and its separation effectiveness [18].

Magnetic beads provide easy separation and localization of target analytes by an external magnet, fast immunoreactions between antigen and antibody and low nonspecific binding by surface modification [19-21]. To connect the magnetic beads with the specific antibody, we can depend on the conjugation between the biotin which can be immobilized on the surface of the magnetic beads and streptavidin modified with the antibody. The biotin-streptavidin bond resulting from this conjugation recorded as a hard bond which allows forming rigid biotin-binding proteins and creating a suitable strategy to modify the immunosensor using the magnetic beads [22-26].

As a result, the magnetic beads have been used to determine a huge various rang of analytes like prostate specific antigen [27], Polychlorinated Biphenyl [28], pseudorabies and avian influenza viruses [29].

Transferring the chemical changing into a signal which is able to record and notice by human is very sensitive and critical point in the immunosensor design and fabricates process. There are two types of detectors commonly used in the immunosensor the optical and electrochemical transducers.

As well known, electrochemical analysis is considered as the best chose for a sensitive and clean analytical method. In addition of that the electrochemical methods offer the advantages of great speed and simplicity, low cost and relatively short analysis time as compared to other analytical techniques. Because of that, electrochemical detectors are an active area of research [30-32]. The function of the electrochemical transducer base on the detection of the product formed from the reaction of the substrate with the enzyme on the surface of the second antibody in the case of enzyme immunoassay. This case put strict conditions on the selected substrate, which should be electrochemical inactive within wild potential range. On the contrary, the product must be electrochemical active to obtain high signal on the surface of the electrode. In the case of using alkaline phosphatase as an enzyme conjugated with the second antibody [33-35], α-naphthyl phosphate was used as a stable and inexpensive substrate in addition of being electrochemically inactive over a wide range of potentials [36-38]. On the other hand, α-naphthol formed as a product of the enzymatic reaction is electrochemically active and its hydroxyl group represents an active function group to electrochemical detection [39].

Carbon material electrodes are considered one of the most common choses in electrochemical analysis. Advantages like easy fabrication, cheap cost of manufacturing, and highly selectivity make the carbon electrodes an optimum tool in the research area. There are various types of carbon electrodes include of carbon paste electrode [40], glassy carbon electrode [41], screen printed carbon film [42-43], carbon powder [44], Carbon nanotubes [45] and Graphite pencil electrode [46]. As noticed the graphite pencil electrode represents an easy use, inexpensive and disposable tool which led us to consider it as more favorite selection in comparison with the more expensive commercial carbon electrodes [47]. Different kinds of analytes were successful determined by the graphite pencil electrode including the detection of vitamin C content of commercial juice [48], caffeine [49] and detection of ozone in water [50]. However, the detection of the biological analytes by using the graphite pencil electrode was done and give the opportunity to be used in the different biochemical analysis, For example DNA and RNA segments [51-53], hemoglobin detection [54] and the salicylic acid in plant material

[55]. The efficiency of the graphite pencil electrode can be increased by many strategies, one of them using the nanoparticles to modify it. According to this technique, graphite pencil electrode modified with palladium nanoparticles used to determine the hydrogen peroxide [56]. Furthermore, layer-by-layer gold nanoparticles modified graphite pencil electrode used to sense the same target using horseradish peroxidase as a labeled enzyme [57]. In general, the graphite pencil electrode characterizes with the simplicity of the modification of the surface, for example the gold nanoparticles modified is needed just to immerse the bare of electrode in the gold nanoparticle solution following by heating which used successfully to determine different kinds of the anlytes [58].

2.3 Experimental section

2.3.1 Materials and methods

2.3.1.1 Reagents

All solutions were prepared using double distilled water. Phosphate buffered saline (PBS), Tris-hydrochloride, α-naphthyl phosphate disodium salt, α-naphthol, albumin from bovine serum (BSA), Carbon glassy powder, 0.05 microns gamma alumina powder and glassy carbon powder were obtained from Sigma, Micro particle (magnetic, streptavidin coated) and sodium hydroxide was purchased from Sigma-Aldrich. Lithium chloride was supplied from Fluka AG (Chemische Fabrik CH9470, Buch). Anti-rabbit IgG biotin conjugate, Rabbit IgG and anti-rabbit IgG alkaline phosphatase conjugate was obtained from Thermo Scientific. Graphite carbon powder was obtained from Fisher.

2.3.1.2 Apparatus and procedures

Voltammetry measurements were performed with an electrochemical workstation (CHI1140A, CH Instruments Inc, Austin, TX, USA). The Ag/AgCl reference electrode (in 3 M KCl, CHI111, CH Instruments Inc) and platinum wire counter electrode (CHI115, CH Instruments Inc).The magnetic bead assays and separations were performed on a MCB 1200 Biomagnetic processing platform (Sigris Research).

2.3.2 Procedures

2.3.2.1 Electrochemical detection of α-naphthol

Three electrodes system was applied to detect a pure α-naphthol dissolved in tris buffer (0.1 M, pH 9.8). The pastes for both glassy carbon paste electrode and graphite carbon paste electrode were prepared by mixing 70:30 (w/w) carbon powders to mineral oil. The GCE was polished with 0.05 microns gamma alumina applied on a plate and washed with deionized water before the measurement. Silver/silver chloride (Ag/AgCl) in potassium chloride solution was used as a reference electrode while platinum wire serves as the counter electrode. All the three electrodes were put in the glass cell with 2 ml of the solution. Both the cyclic and square wave voltammetry techniques were used in the work. As mentioned before the square wave voltammetry was considered to test the optimization process and calibration curve.

2.3.2.2 Preparation of antibody-conjugated magnetic beads

The magnetic beads preparation was carried out on an MCB 1200 Biomagnetic processing platform using a modified procedure recommended by Bangs Laboratories (Tech Note 101). Briefly, 2.5μL (i.e., 25 μg) of streptavidin-coated magnetic beads were transferred into a 1.5 mL centrifuge tube; Then, 95 μL of the TTL buffer (100 mM Tris-HCl, pH 8.0, 0.1% Tween, and 1 M LiCl) was added and mixed for 1 min with a speed of 0.5 rps. Application of the magnetic field (to the side of the centrifuge tube) attracted the beads to the sidewall. After the solution became clear, it was carefully removed with a pipette. The washing step was repeated once. The beads were then suspended in 16.67 µL of TTL buffer. Then, 8.33 μL of biotinylated antibody (anti-rabbit IgG) was added to yield a final antibody concentration of 100 μg/ml. The mixture was incubated for 30 min with gentle mixing. After magnetic separation, the antibody-magnetic beads were washed twice with 95 µL of TT buffer (250 mM Tris-HCl, 0.1% Tween 20) and suspended in 45 μL of TTL buffer.

2.3.2.3 Sandwich immunoassay

The selected amount of rabbit IgG was then added and mixed for 30 min. Following their magnetic isolation, the magnetic beads bearing the immunocomplex were washed twice with 95 µL of TT and resuspended again in 40 μL of TTL buffer. Subsequently, 10 µL of alkaline phosphatase conjugated-anti-rabbit IgG was added to yield a final antibody concentration of 10 μg/ml, and mixed for 30 min. The resulting sandwich-conjugated microspheres were washed three times with 95 µL of TT. Then, 50 µL of tris buffer (0.1 M, pH 9.8) containing 2 mM of α-naphthyl phosphate disodium salt was added. After a 5 min mixing and a magnetic separation, the resultant solution was transferred into 0.5 mL of centrifuge tube and diluted it to 250 µL with adding tris buffer pH 9.8. Next, the diluted solution was transferred to the 0.2 mL electrochemical cell to measure the electrochemical signal by square wave voltammetry technique. The electrochemical cell contained a bare graphite pencil electrode as a working electrode, a Pt wire counter electrode, and Ag/AgCl (sat. KCl) reference electrode Control experiments were performed in a similar fashion but without adding the antigen.

2.4 Results and discussion

2.4.1 The cyclic voltammetric measurement of α-naphthol

To scan the α-naphthol electrochemical properties, the cyclic voltammetry technique was applied by using the graphite pencil electrode as a working electrode, a Pt wire counter electrode, and Ag/AgCl (sat. KCl) reference electrode. Starting from low potential, the voltammogram shows noticeable oxidation current increase starting from 0.1V. The highest oxidation current signal was recorded at 0.3 V (Figure 2.6).

2.4.2 Screening of different carbon electrodes

Four carbon electrodes including of glassy carbon paste electrode, graphite carbon paste electrode, pencil electrode and glassy carbon electrode were studied by calculating the ratio between the signal and the electrode area (Figure 2.7). Graphite pencil electrode showed the highest ratio due to its better electron transfer properties in comparison with the other electrodes tested. Depending on the results and its high stability and precision compared to the other solid electrodes, graphite pencil electrode was selected as a working electrode for the following experiments.

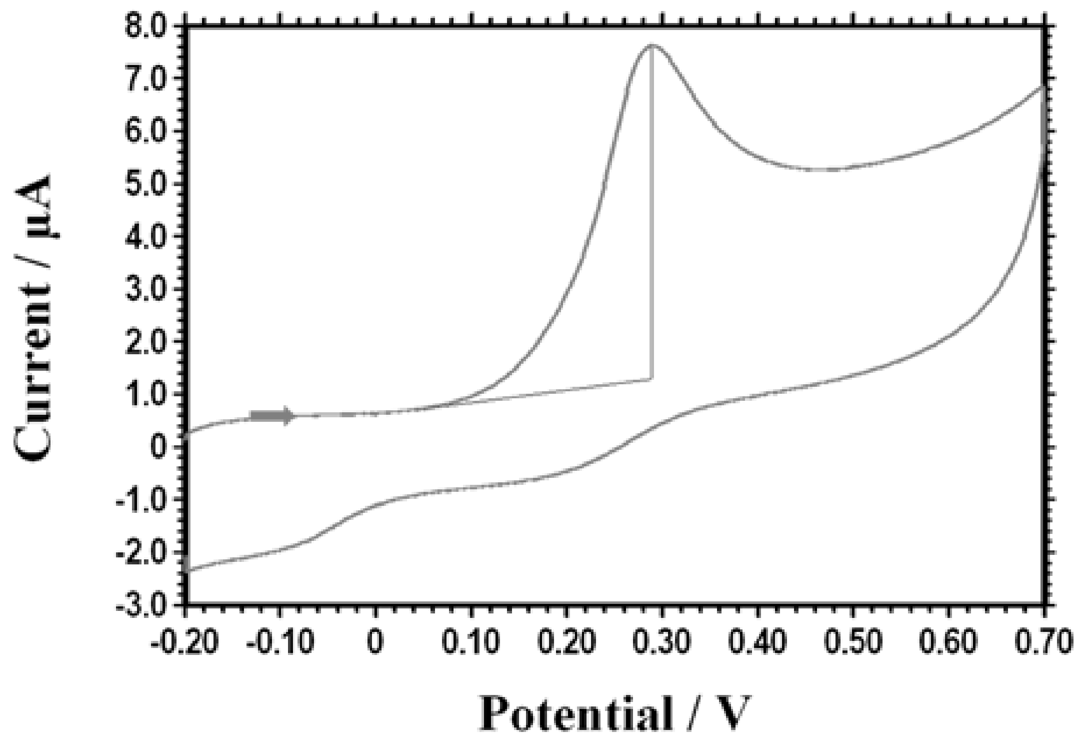

Figure 2.6: Cyclic voltammogram of the graphite pencil electrode in 0.1 M Tris buffer (PH 9.8) containing 30 μM α-naphthol.

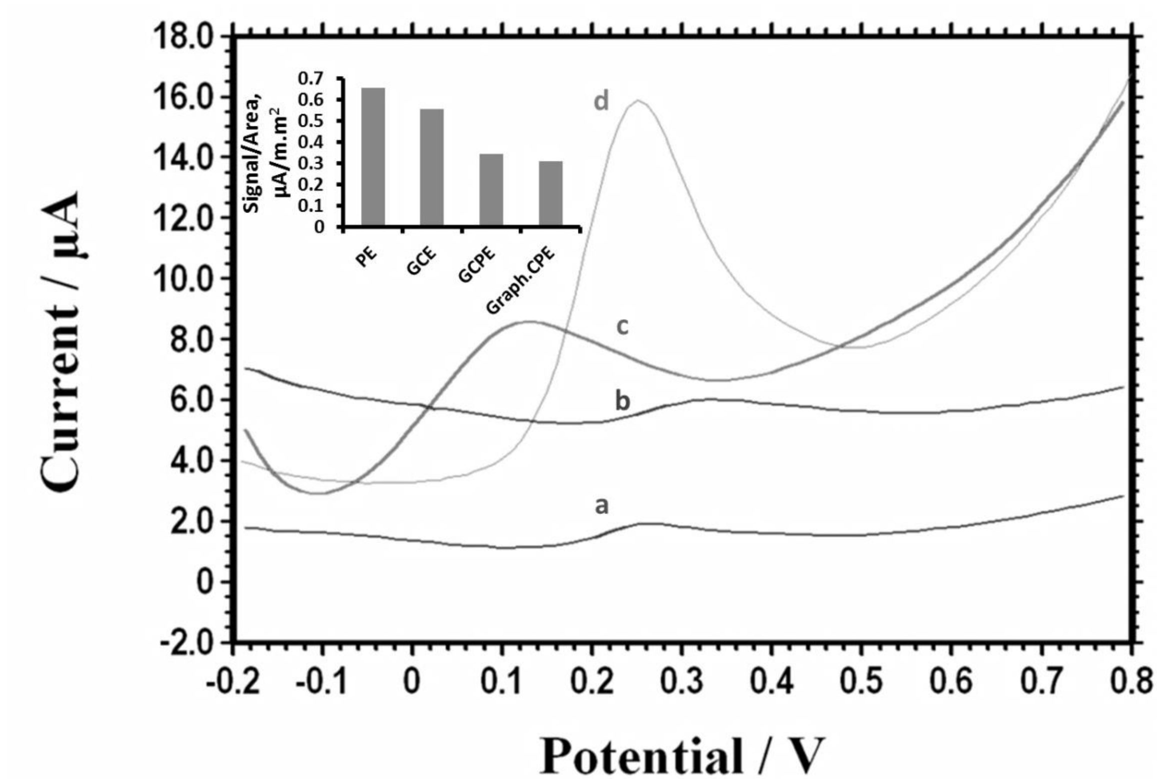

Figure 2.7: Square wave voltammograms of different electrodes in 0.1 M Tris buffer (pH 9.8) containing 30 µM α-naphthol. (a) Glassy carbon paste electrode (surface area 2.00 mm^2), (b) graphite carbon paste electrode (surface area 2.00 mm^2), (c) glassy carbon electrode (surface area 7.06 mm^2), (d) graphite pencil electrode (surface area 15.70 mm^2). The inset represents the Signal/Area for each electrode, (PE) pencil electrode, (GCE) glassy carbon electrode, (GCPE) glassy carbon paste electrode, (Graph.GPE) graphite carbon paste electrode.

2.4.3 Optimization of the electrochemical parameters

2.4.3.1 Optimization of potential increment

Potential increment (Increment E) was optimized by studying the range from 5 to 25 mV (Figure 2.8). The signal showed non acceptable change shape and a slight change of response values above 15 mV. Therefore, the value of 15 mV was chosen depending on the response and the shape of the peak.

2.4.3.2 Optimization of Amplitude

The amplitude was recorded from 60 to 140 mV and it was noticed that the signal increases by increasing the amplitude and hits a highest response at 100 mV with slightly decrease was recorded after the peak. The value of 100 mV was chosen as the optimum amplitude depending on the signal response and acceptable shape (Figure 2.9).

2.4.3.3 Optimization of Frequency

The frequency was studied in the range from 10 to 50 Hz. It is known that the response of the signal increases as the frequency increases, yet this increase at the same time affects the shape of the signal at low concentrations and reduces the possibility of recording their responses. As our work was focusing to enhance both the limit of quantification and limit of detection, we chose the value of 30 Hz to apply as the optimum frequency depending on the signal response and acceptable shape (Figure 2.10).

2.4.3.4 Optimization of accumulation potential

Accumulation potential study was done by testing -200, -100, 0, 100 and 200 mV (Figure 2.11). As it is clear from the inset of (Figure 2.11), the accumulation potential affects negatively the response. According to the graph, we noticed that no signal is recorded due to the accumulation potential of 200 mV because it is in the range of oxidation potential of α-naphthol.

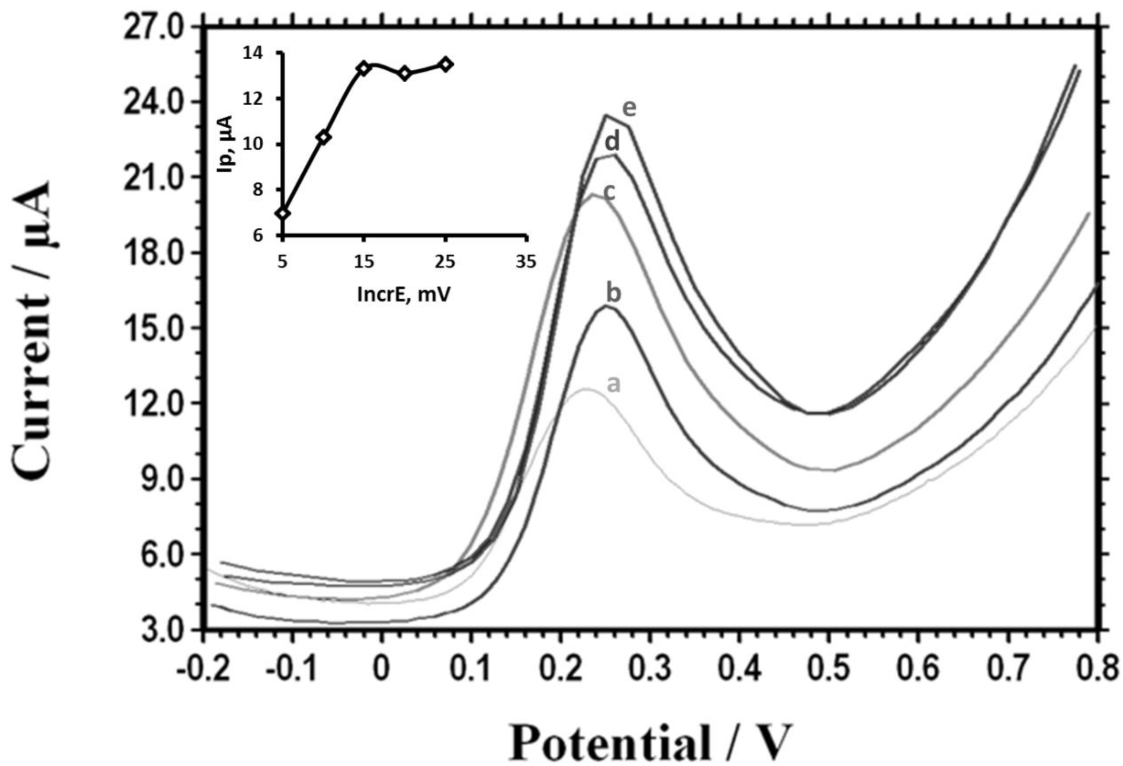

Figure 2.8: Square wave voltammograms of pencil electrode in 0.1 M Tris buffer (pH 9.8) of different Incr. E containing 30 μM α-naphthol. Increment potentials: (a) 5, (b) 10, (c) 15, (d) 20, (e) 25 mV. The inset represents the corresponding plot.

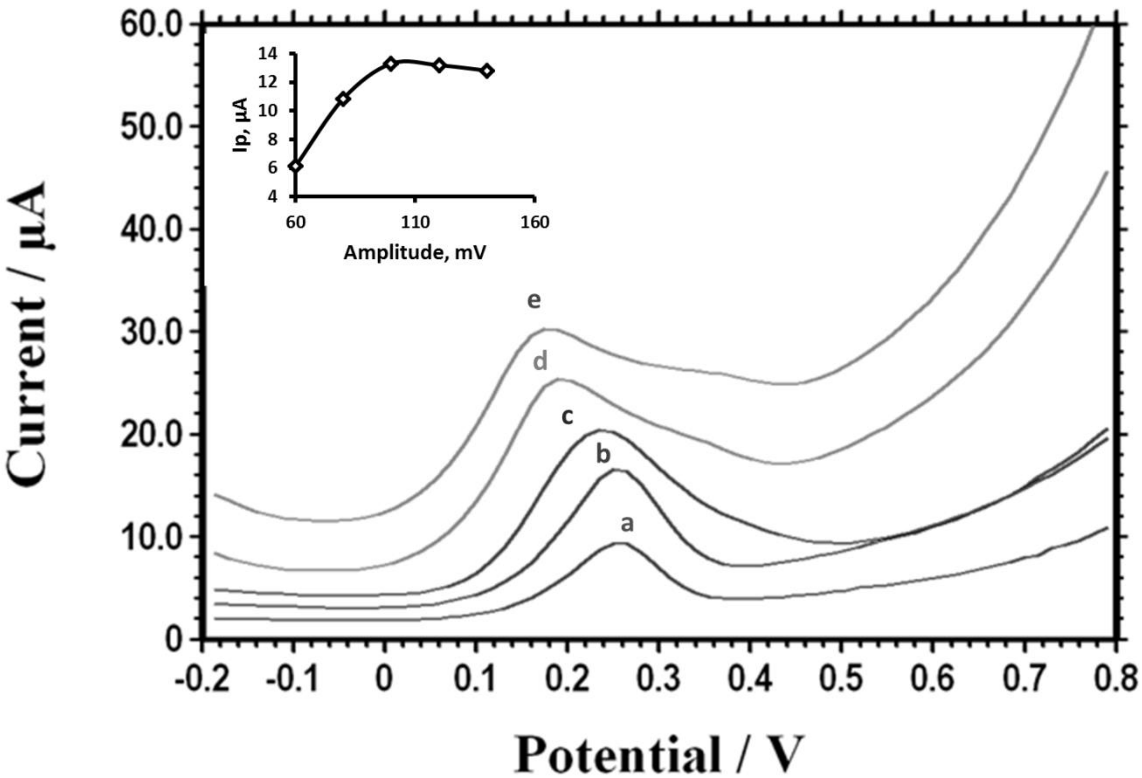

Figure 2.9: Square wave voltammograms of pencil electrode in 0.1 M Tris buffer (pH 9.8) of different Amplitude containing 30 µM α-naphthol. Amplitude: (a) E 60, (b) 80, (c) 100, (d) 120, (e) 140 mV. The inset represents the corresponding plot.

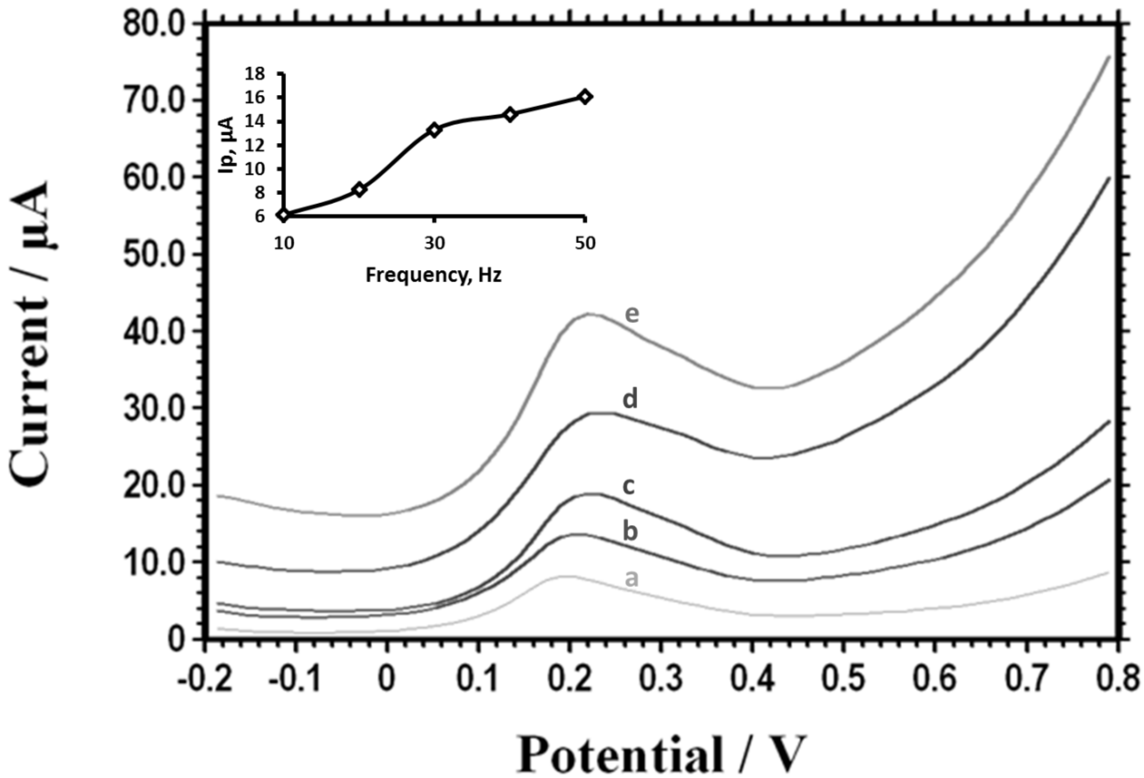

Figure 2.10: Square wave voltammograms of pencil electrode in 0.1 M Tris buffer (pH 9.8) of different frequency containing 30 µM α-naphthol. Frequency (a) 10, (b) 20, (c) 30, (d) 40, (e) 50 Hz. The inset represents the corresponding plot.

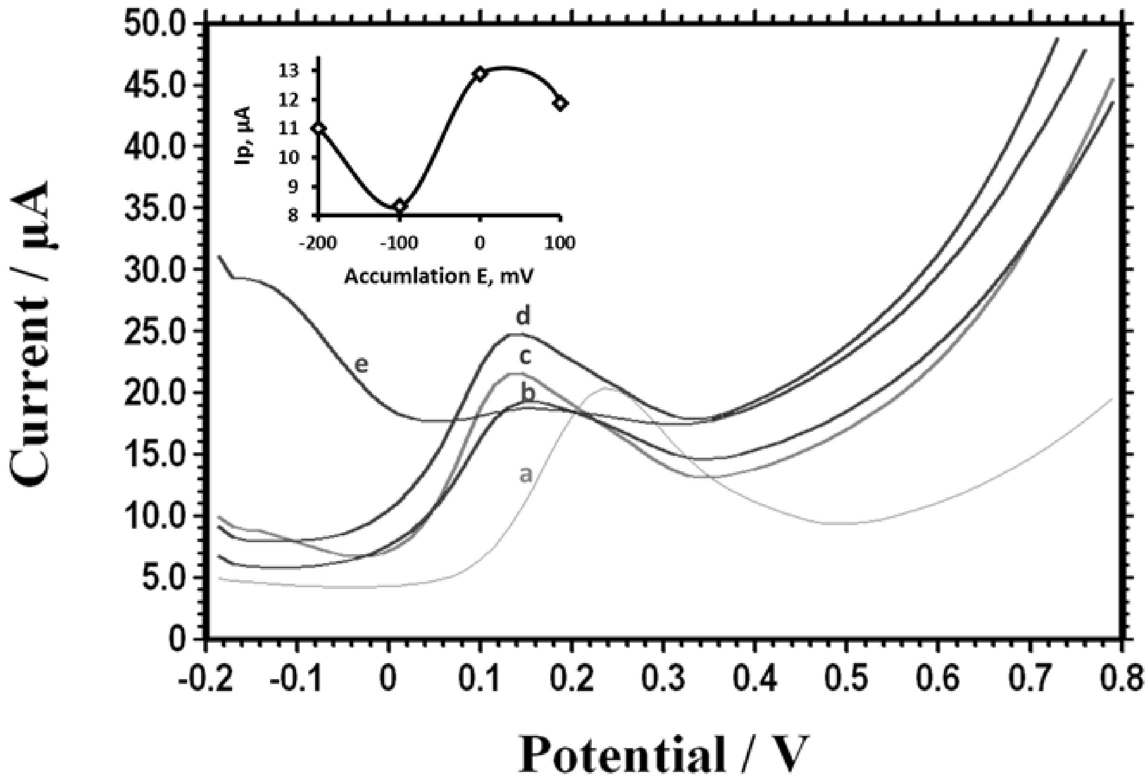

Figure 2.11: Square wave voltammograms of pencil electrode in 0.1 M Tris buffer (pH 9.8) of different accumulation E containing 30 µM α-naphthol. Accumulation E (a) 0, (b) -0.1, (c) 0.1, (d) -0.2, (e) 0.2 V. The inset represents the corresponding plot.

2.4.4 Optimization of immunosensing parameters

2.4.4.1 Optimization of the magnetic beads amount

The optimization process included a test of five amounts of the magnetic beads which were 10, 25, 50, 75 and 100 µg in the case of presence and absence of the target material (Figure 2.12). The signal rises, directly proportional to the increase in the amount of magnetic beads in the case of presence of the antigen (part A Figure 2.12). However, in the case of absence of the antigen, slightly remarkable signals for 10 and 25 µg added from the magnetic beads were recorded. For higher amounts, appearance of increasingly signal proportional to the amount was observed which can be explained by non-specific adsorption effect of alkaline phosphatase conjugated anti-rabbit IgG molecules on the surface of the magnetic beads (part B Figure 2.12). The amount of 10 µg of magnetic beads was chosen depending on the higher signal recorded and slightly remarkable background.

2.4.4.2 Optimization of the concentration of the biotinylated anti-Rabbit IgG

Four concentrations were tested including 10, 25, 50 and 100 µg/ml of anti-rabbit IgG biotinylated (Figure 2.13). The experiments did not show any observed remarkable signal increase with increasing the concentration in the case of presence of the antigen (part A Figure 2.13). In the case of absence of the antigen, it was remarkable that low concentrations of anti-rabbit IgG caused non-specific absorption effect on the non-covered parts of the magnetic beads by alkaline phosphatase conjugated anti-rabbit IgG molecules which led to observed signal. Further, slightly low signal was recorded for high concentration of 100 µg/ml of anti-rabbit IgG biotinylated added (part B Figure 2.13). Depending on the value of the signal and noise responses, the concentration of 100 µg/ml was chosen for the following experiments as an optimized condition.

2.4.4.3 Optimization of the concentration of the alkaline phosphatase conjugated anti-Rabbit IgG

Three concentrations were studied to optimize the concentrations of alkaline phosphatase conjugated anti-rabbit IgG include 1, 10, 50 µg/ml (Figure 2.14). As it appears in (part A Figure 2.14) the signal increases by increasing the concentration in the case of presence the target. For both concentrations of 1 and 10 µg/ml a slightly background was noticed when the target is absent in the experiment cell, but in the case of high concentration of 50 µg/ml a clear signal was recorded due to the non-specific absorption effect (part B Figure 2.14). However, the concentration of 10 µg/ml is chosen as an optimum concentration according to its signal response and low background recorded.

2.4.5 Electrochemical determination of rabbit IgG at graphite pencil electrode surface

The calibration curve of α-naphthol was plotted by testing different concentrations of rabbit IgG (Figure 2.15). The equation form adapted in the calibration of the inset of (Figure 2.15) is I µA = 5.572 log [Rabbit IgG] ng/ml + 6.328 and $R^2 = 0.9813$. The limit of quantification of the developed sensor is 1 ng/ml and the detection limit (at 3σ) is 0.16 ng/ml.

The reproducibility of the sensor was studied by testing replicate measurements and the relative standard deviation was determined 3.8 %.

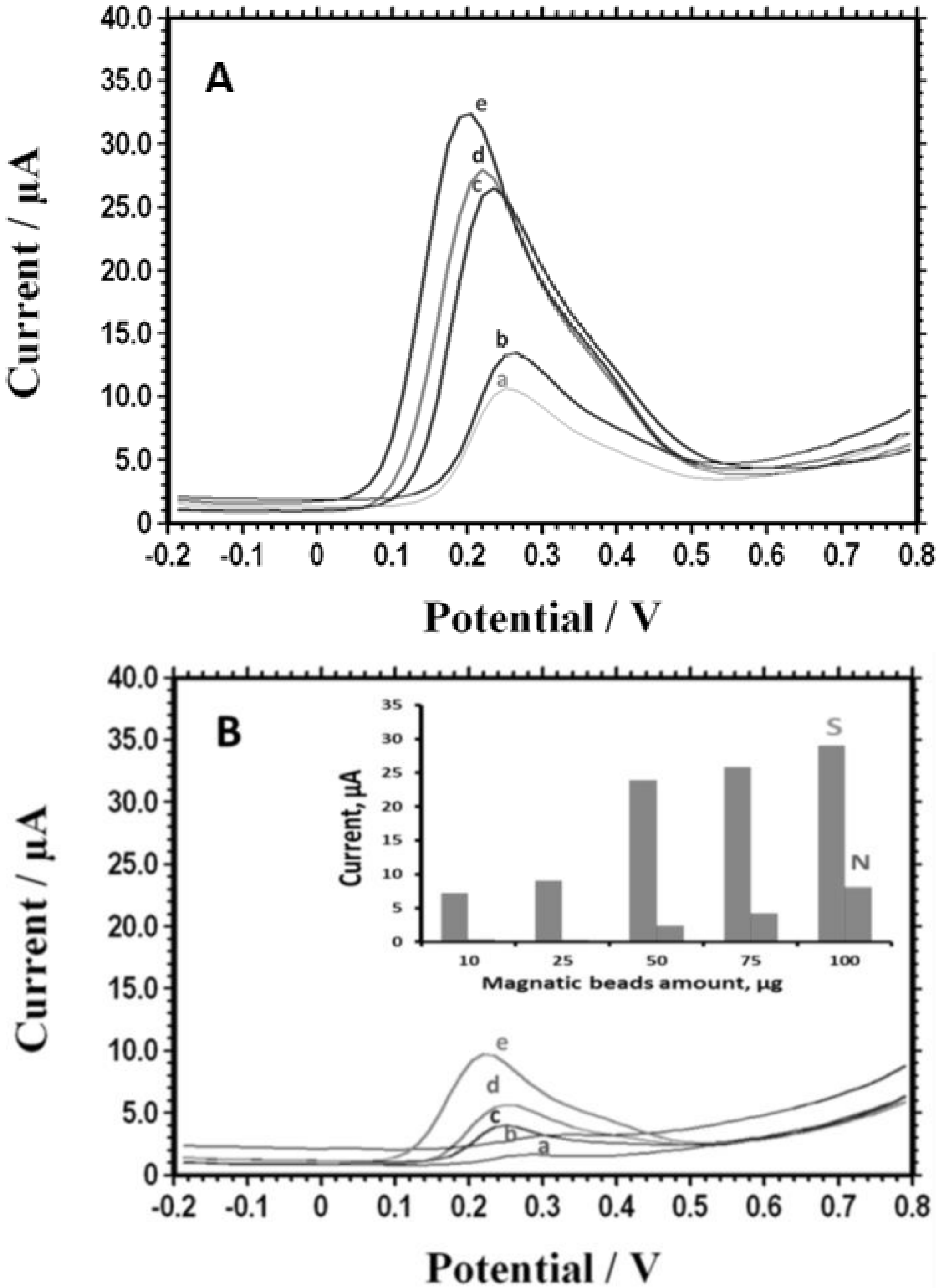

Figure 2.12: Square wave voltammetric signals for immunosensor using different amount of magnetic beads for the presence (A) and absence (B) of 10 ng/ml from rabbit IgG. Amount of magnetic beads (a) 10, (b) 25, (c) 50, (d) 75, (e) 100 µg. The inset represents the histogram of signal (S) to noise (N) ratio vs. the magnetic beads amount.

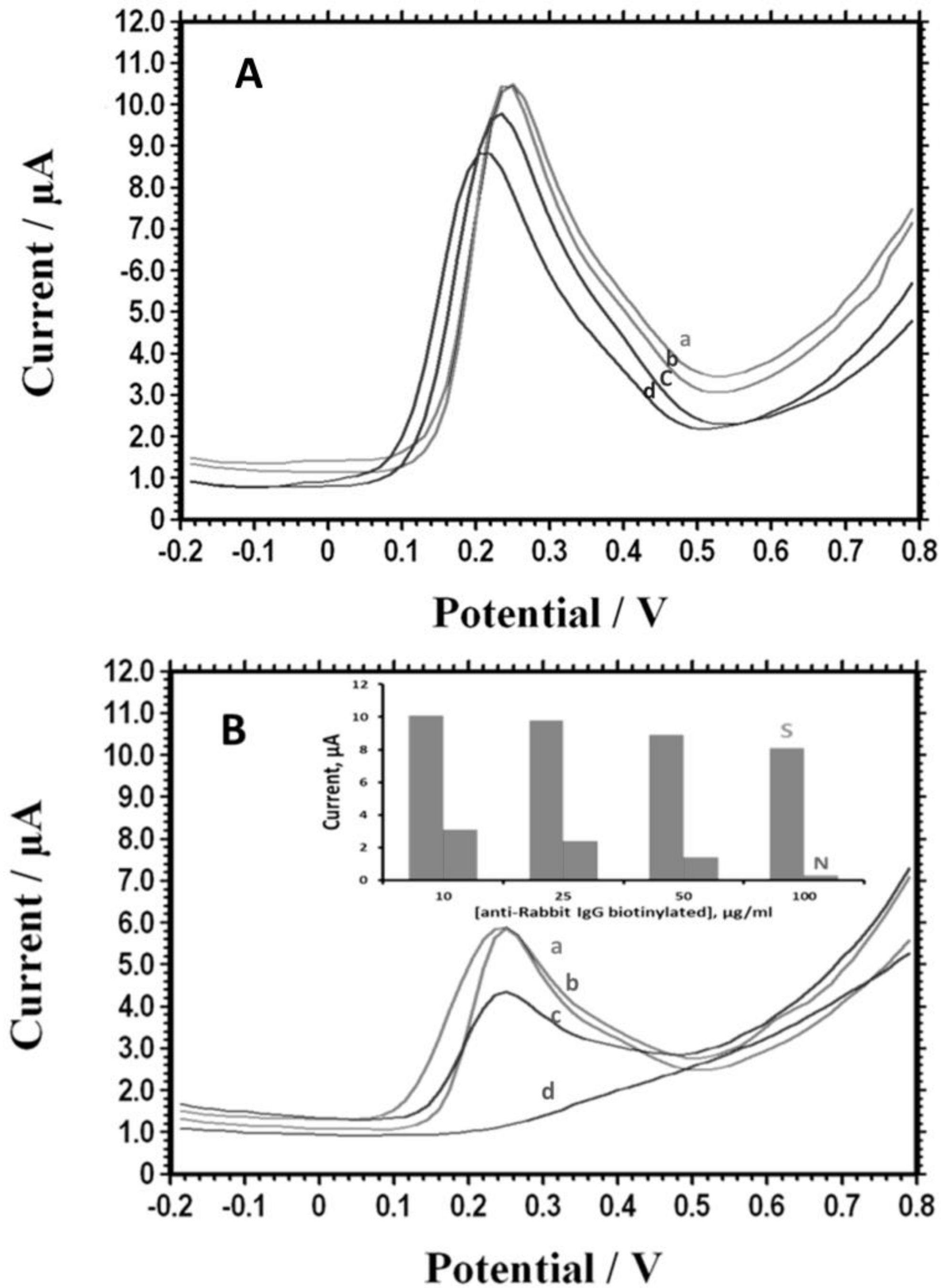

Figure 2.13: Square wave voltammetric signals for immunosensor using different concentrations of anti-Rabbit IgG biotinylated for the presence (A) and absence (B) of 10 ng/ml from Rabbit IgG. [Anti-Rabbit IgG biotinylated] (a) 10, (b) 25, (c) 50, (d) 100 µg/ml. The inset is the corresponding histogram of the current to signal (S) to noise (N) ratio vs. the amount of the biotinylated anti-Rabbit IgG.

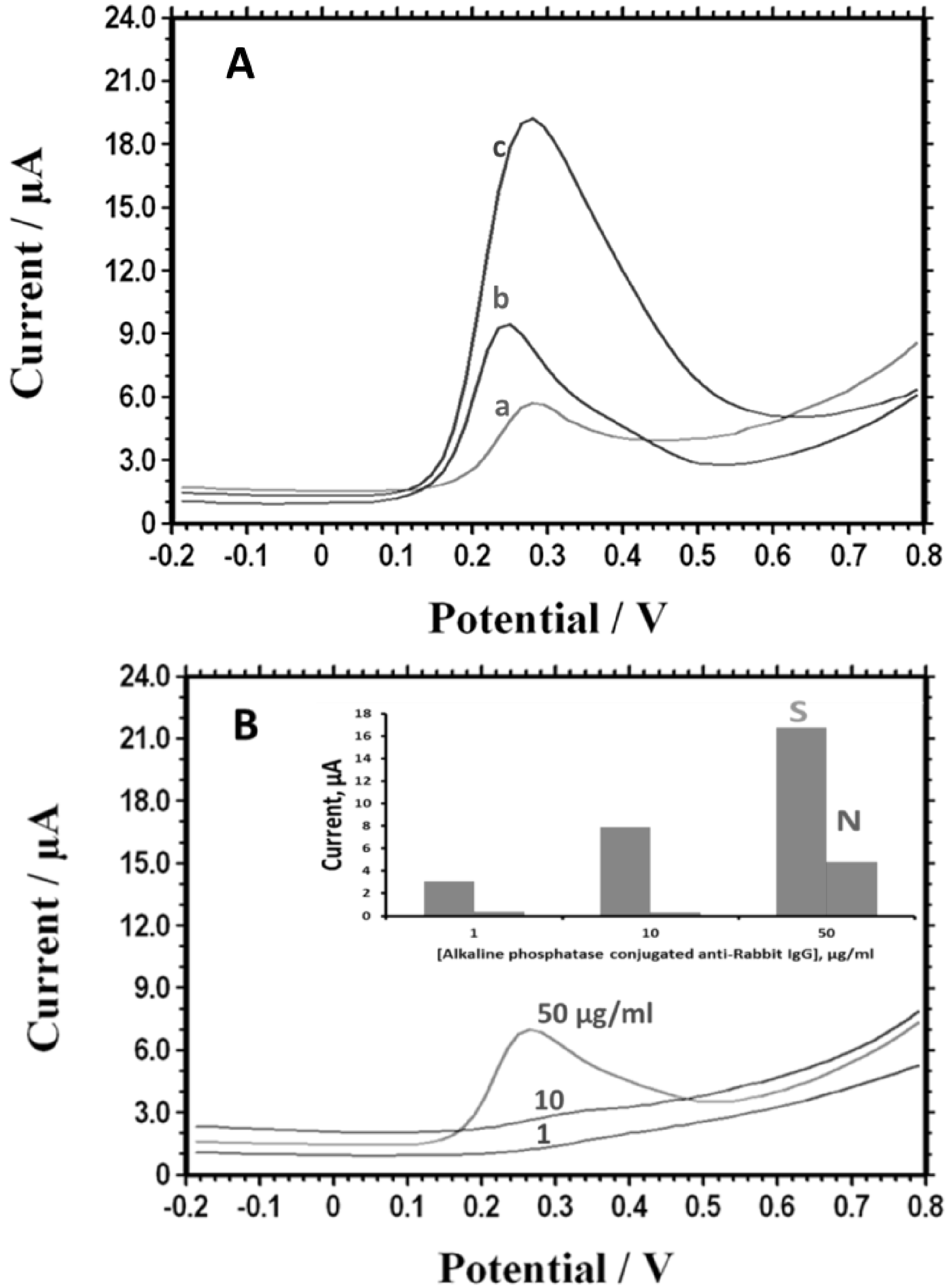

Figure 2.14: Square wave voltammetric signals for immunosensor using different concentrations of alkaline phosphatase conjugated anti-Rabbit IgG for the presence (A) and absence (B) of 10 ng/ml from Rabbit IgG. [Alkaline phosphatase conjugated anti-Rabbit IgG] (a) 1, (b) 10, (c) 50 µl/ml. The inset histogram represents the current to signal (S) and noise (N) responses.

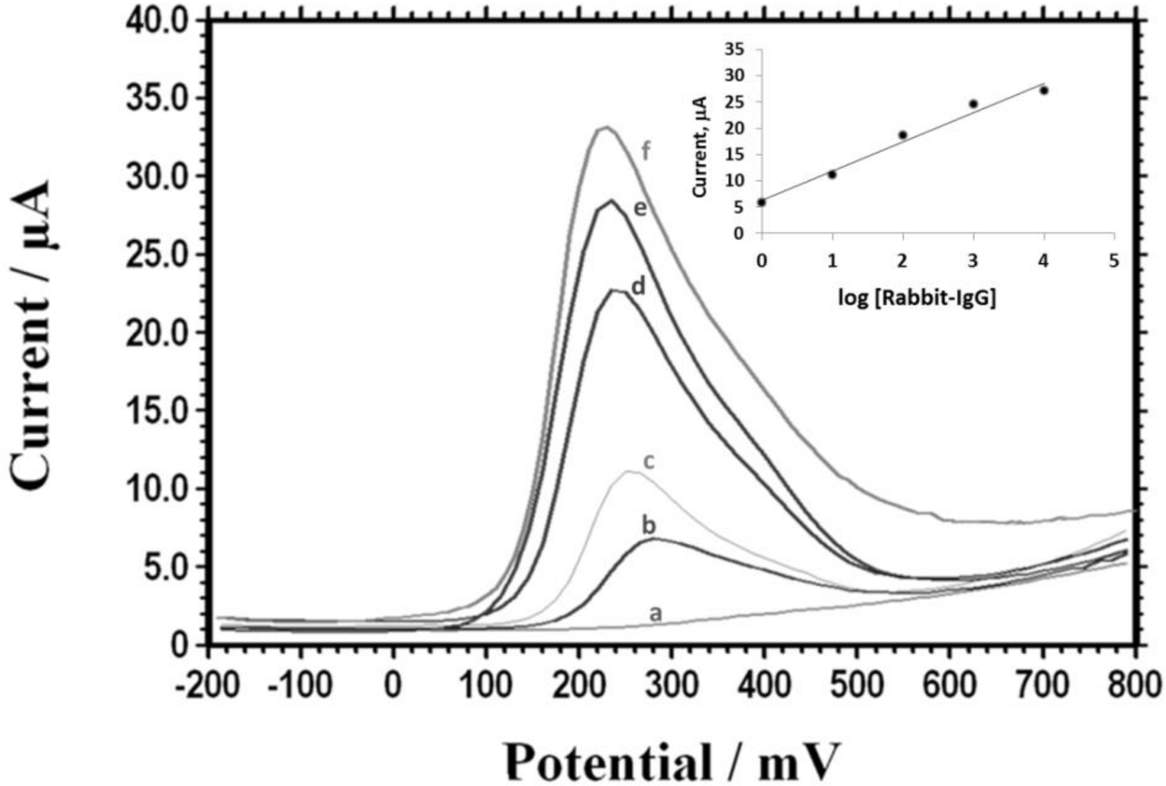

Figure 2.15: Rabbit-IgG concentrations dependence square wave voltammograms of pencil electrode in 0.1 M Tris buffer pH (9.8). The amount of magnetic beads = 25 µg, [anti-Rabbit IgG biotinylated] = 100 µg/ml, [Alkaline phosphatase conjugated anti-Rabbit IgG] = 10 µg/ml, [α-naphthyl phosphate disodium] = 2 mM. The concentration (a) blank, (b) 1, (c) 10, (d) 100, (e) 1000, (f) 10000 ng/ml. The inset represents the corresponding calibration plot.

2.5 Conclusion

On balance, the work achieved in this chapter based on using the magnetic beads based immunoassay connected with electrochemical detector to fabricate an immunosensor designed to detect rabbit IgG antigen. Streptavidin-coated magnetic beads type is used in the fabrication. The streptavidin-coated magnetic beads are reacted with biotinylated anti-rabbit IgG. Then a sample contained rabbit IgG target was added, followed with alkaline phosphatase conjugated-anti-rabbit IgG adding to form the sandwich compound. α-naphthyl phosphate is used as a substrate and reacts on the phosphatase enzyme surface and produce α–naphthol as a product which can be detected using electrochemical transducer. The three electrode system is applied to detect of α–naphthol testing different kind of carbon electrodes, and graphite pencil electrode is chosen among the tested electrode. The cyclic voltammetry technique is run for qualitative detection while square wave voltammetry technique is used for quantitative detection. To enhance the signal produced by square wave voltammetry, all the effect parameters were optimized including potential increment, amplitude, frequency and accumulation potential. The optimized parameters adopted are 0.015 V increment potential, 0.1 V amplitude, 30 Hz frequency and without applying accumulation potential.

To enhance the performance of biosensor the effect of different concentrations of components were studied and optimized and as the result, the final amount and concentrations depended are the magnetic beads amount is 25 µg, the concentration of the biotinylated anti-Rabbit IgG is 100 µg/ml and the concentration of the alkaline phosphatase conjugated anti-Rabbit IgG is 10 µg/ml. The limit of quantification of developed sensor is 1 ng/ml and the detection limit (at 3σ) was estimated to be 0.16 ng/ml.

CHAPTER 3

Strip based dry immunosensor

3.1 Introduction

3.1.1 Definition

Lateral flow tests or lateral flow immunochromatographic assays are a simple device intended to detect the presence (or absence) of a target analyte in sample (matrix). Most commonly these tests are used for medical diagnostics either for home testing, point of care testing, or laboratory use. The benefits of immunochromatographic tests include: (1) user-friendly format (2) very short time (generally less than ten minutes) for a test result (3) less interference due to the chromatographic separation (4) long-term stability over a wide range of climates (5) relatively inexpensive to manufacture. These features make strip tests ideal for applications such as home testing.

3.1.2 Lateral flow test design

Lateral flow strip consists from four pads: (1) sample pad (2) conjugate pad (3) Nitrocellulose pad which contains the test and control zones (4) absorption pad (Figure 3.1).

Four kinds of membranes were used for the lateral flow strip fabrication including cellulose ester membrane for the control-test pad, glass fibers for conjugate pad, non-woven cotton fiber material using as a sample and absorption pad, and plastic Adhesive backing for assembling all the other membranes (Figure 3.2).

3.1.3 Mechanism of the lateral flow test

In the first step, the labeled anti-body is dispensed on the conjugate pad following with the primary and secondary anti-bodies dispensing on the test and control zones respectively (Figure 3.3). In general, the antigen as a target analyte in the sample will be applied in the sample pad to form a complex compound with the anti-body in the conjugate pad. As a buffer solution was applied on all the strip, the complex compound will move to be captured on the test zone by the primary antibody showing distinctive color or producing measurable product after apply a substrate solution, extra conjugated antibody continue the immigration to captured on the control zone using secondary antibody (Figure 3.4). Getting two color lines in the two zones identifies the presence of the antigen in the sample; on the other hand appearance just one color line on the control zone identifies the absence of the analyte and efficiency of the strip. The possibility of measurement the tension of the color on the two zones and the electrochemical determine of product produced by applied the substrate allows for quantitative measurement. One of the most common applies of the lateral-flow chromatographic strip technique is determine of proteins which opens the way for early diagnosis of cancer diseases synchronizing of increase in the level some types of proteins in the blood. In addition of that, recent nanoparticles applied on the flow chromatographic strip technique improve the activity of the process, enhance the determine ranges, and expend the detected analytes area.

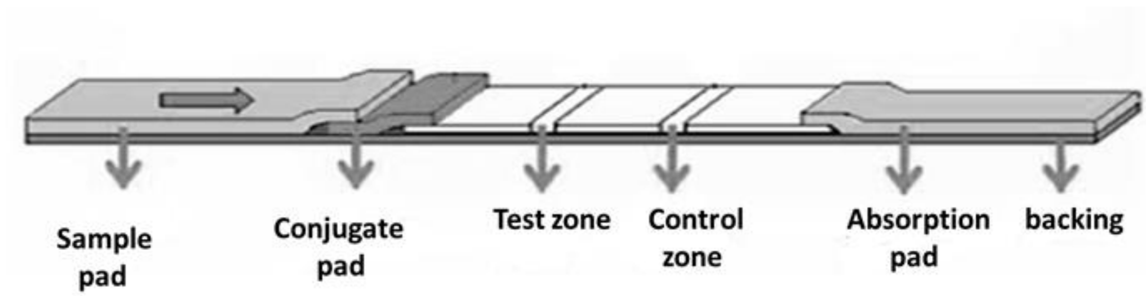

Figure 3.1: The structure of the lateral flow strip.

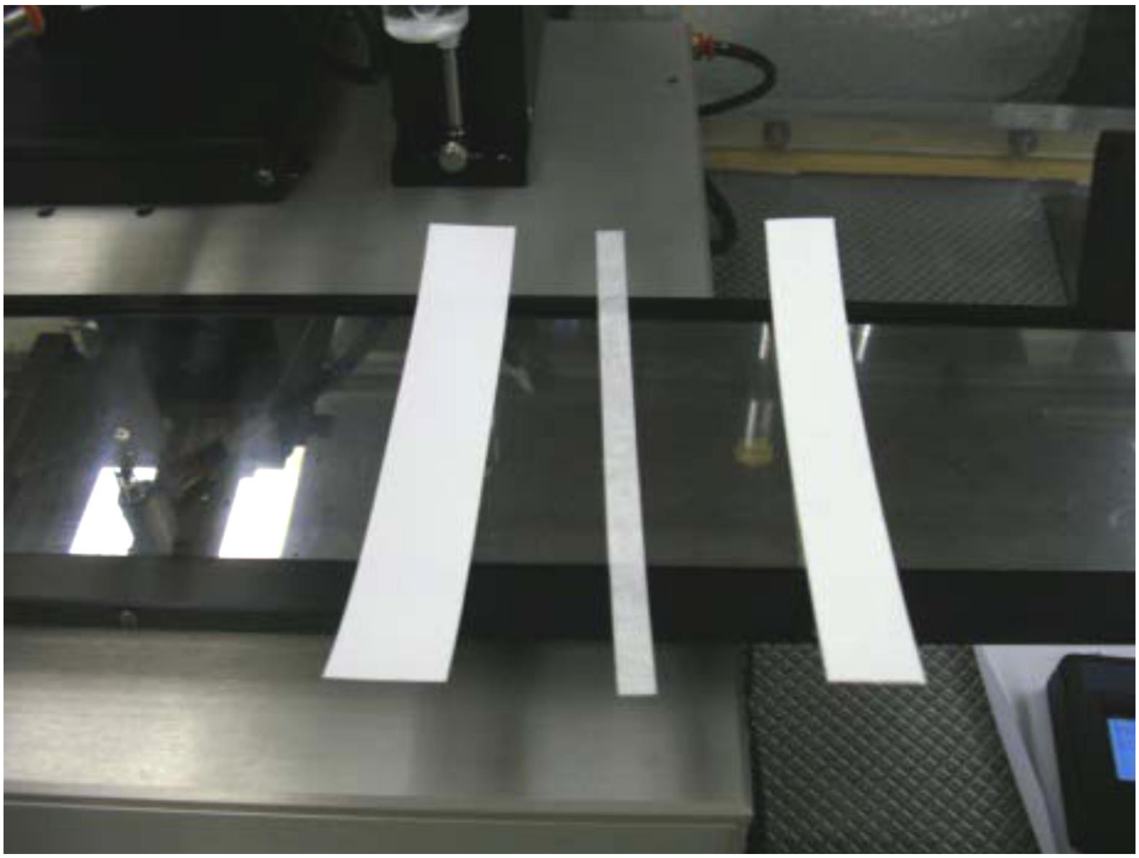

Figure 3.2: The used membranes: from the left cellulose ester membrane, glass fibers and non-woven cotton fiber material.

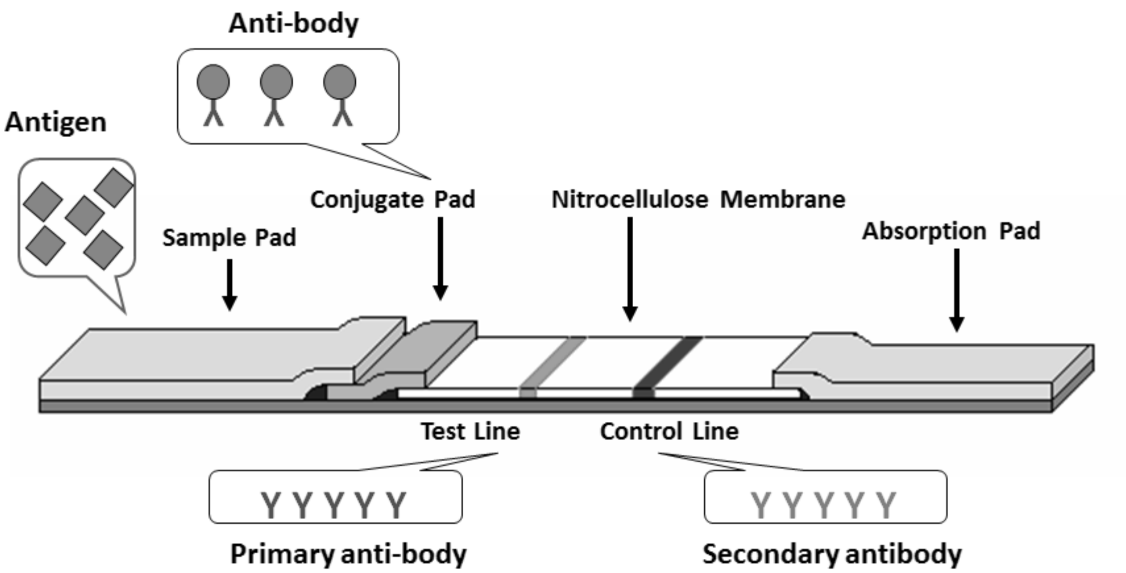

Figure 3.3: A scheme represents the dispensing of the anti-bodies and antigen on the lateral flow strip.

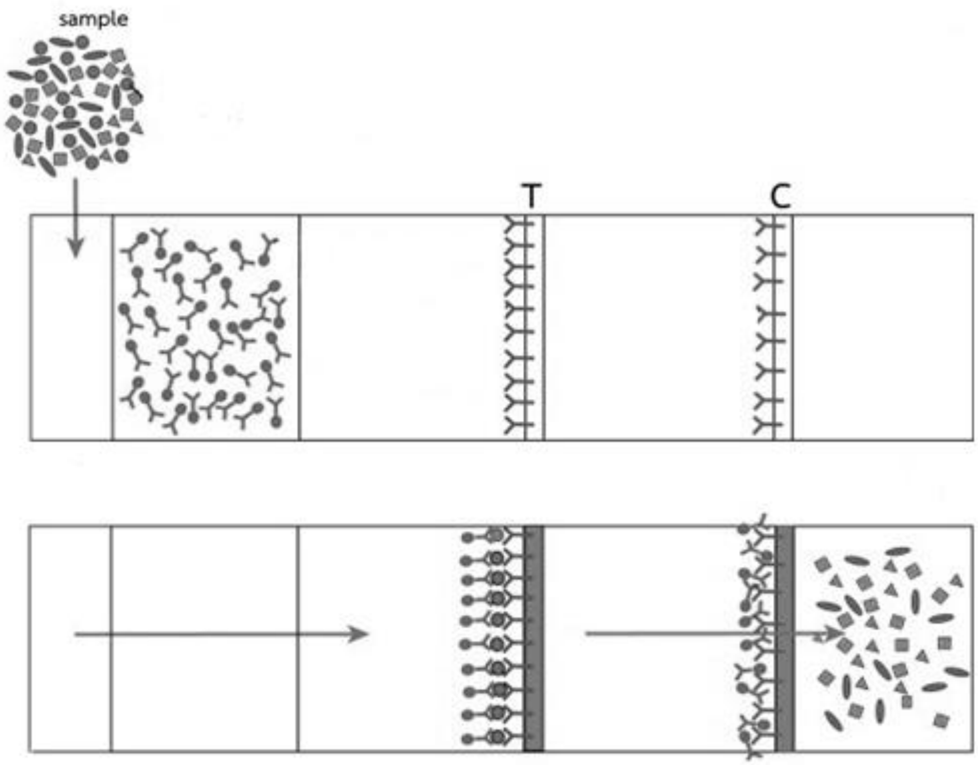

Figure 3.4: A scheme represents the mechanism of the lateral flow test.

3.1.4 The point-of-care enzyme-based dry-reagent strip biosensor

The lateral-flow chromatographic strip is prepared with many chemicals during the fabrication. Antibody-enzyme conjugate compound applied in the conjugate pad, primary antibody is added to the test zone in addition to add secondary antibody to the control zone. The buffer solution is added for the sample pad after we can add the sample which contains the antigen. The antigen reacts with the antibody-enzyme conjugate to form a complex which will be specifically captured by the primary antibody to form sandwich compound, the access from the antibody-enzyme compounds are captured by the secondary antibody in the control zone. In the case, there is no analyte (antigen) in the sample so the capture is happened just on the control pad.

After that the substrate applied on the test strip which will react with the enzyme part on the test zone and control zone. Enzymatic reaction produces a color insoluble product.

We can distinguish three cases in this test: (1) in the case of presence the analyte in the sample so two color lines appear (2) no analyte in the sample, only one color line in the control zone appears (3) damaged strip no lines appear.

The Point-Of-Care AuNP-Based Dry-reagent strip Biosensor to determine the presence of IgG

In this situation, AuNP- antibody conjugate applied instead of enzyme–antibody conjugate in addition no substrate applied here because the gold nanoparticles give the red color for the test and control zones. In our research, AuNPs Goat anti rabbit-IgG conjugate used as an antibody conjugate in the conjugate pad and goat anti-rabbit IgG is dispensed in the control zone to detect rabbit IgG antigen (Figure 3.5).

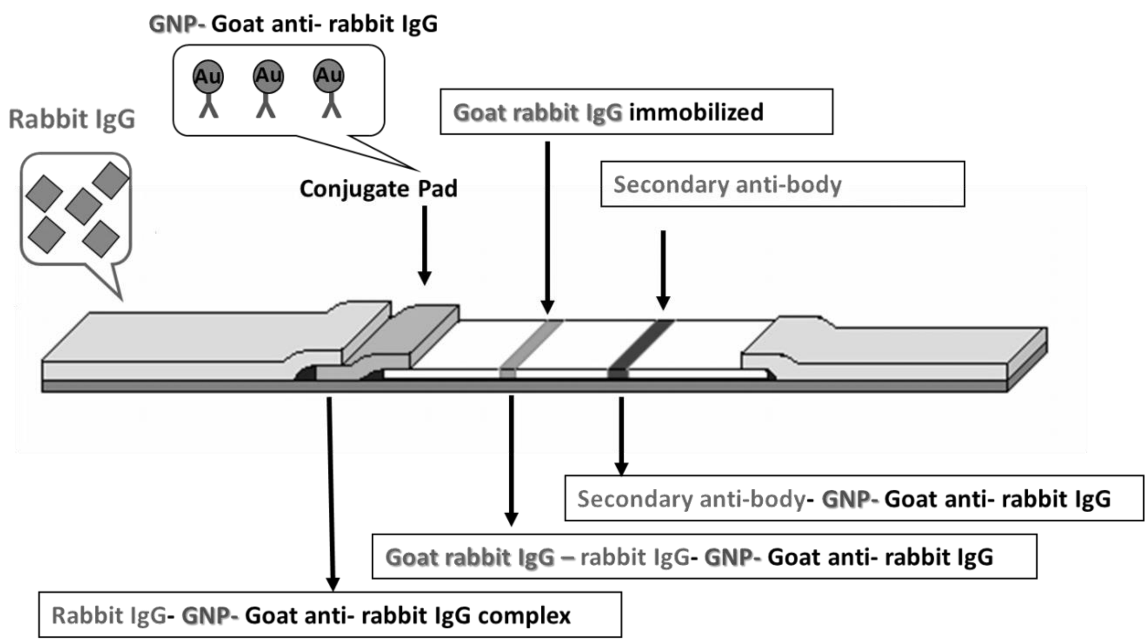

Figure 3.5: A scheme represents the point-of-care AuNPs-based dry-reagent strip biosensor to determine the presence of IgG.

3.1.5 Fabrication of lateral flow test

The fabrication was done by three steps using three machines in the following order: (1) Biojet chemical system (2) Batch laminating system (3) Guillotine cutting system.

1. The biojet chemical system

The biojet chemical system used for the spread the reagent on the membrane by many forms like lines, dots, and aerosol form (Figure 3.6). The membrane is put on the platform and give the position of the membrane on the hand hold controller in addition of the way of dispensing and the number of lines or dots and the kind of the dispenser that we will used. For this system there are three kinds of the dispensers: (1) Air jet dispenser (2) The frontline quanti dispenser (3) The biojet quanti dispenser.

(a) The air jet dispenser: this dispenser used especially for the aerosol spread; this dispenser is used for the antibody-gold nanoparticles conjugate solution.

There are two types of the air jet dispensers: (1) the airjet quanti 3000 is the smallest aerosol dispenser, with a nozzle/needle size of 0.30 mm (2) The airjet quanti 2300 is the second largest aerosol dispenser, with a nozzle/needle size of 0.60 mm.

(b) The frontline quanti dispenser: This dispenser couples a micro-tube dispenser with one or two syringe pumps (based on dispensing rate) linked with a bottle of reagent or ink.

(c) The biojet quanti dispenser: This series of quantitative dispensers couples BioDot's drop-on-demand valve with one or two high-resolution syringe pumps to meter precise amounts of reagent.

Figure 3.6: The biojet chemical system.

2. The batch laminating system

The clamshell designed to assemble a lateral flow assay comprised of materials (typically a nitrocellulose membrane, a sample pad, a conjugate pad, and an absorbent pad) onto an adhesive backing material. Contains top and bottom vacuum nests to hold strip materials in place and is operated manually.

For the assembling membranes: (1) Putting the plastic adhesive backing layer at the top side (2) Putting the membrane by order in the bottom side (3) Closing the clamshell (4) Applying the vacuum. After the assembling process the bulk test strip is achieved (Figure 3.7).

3. The guillotine cutting system

After producing the bulk strip, the strip is cut to smaller pieces by the guillotine cutting system. We can chose the width and number of the pieces requested then we enter the date on the hand hold controller, after that we put the bulk in the specified place, the bulk strip will be cut by sharp blade and get the pieces from the instrument (Figure 3.8).

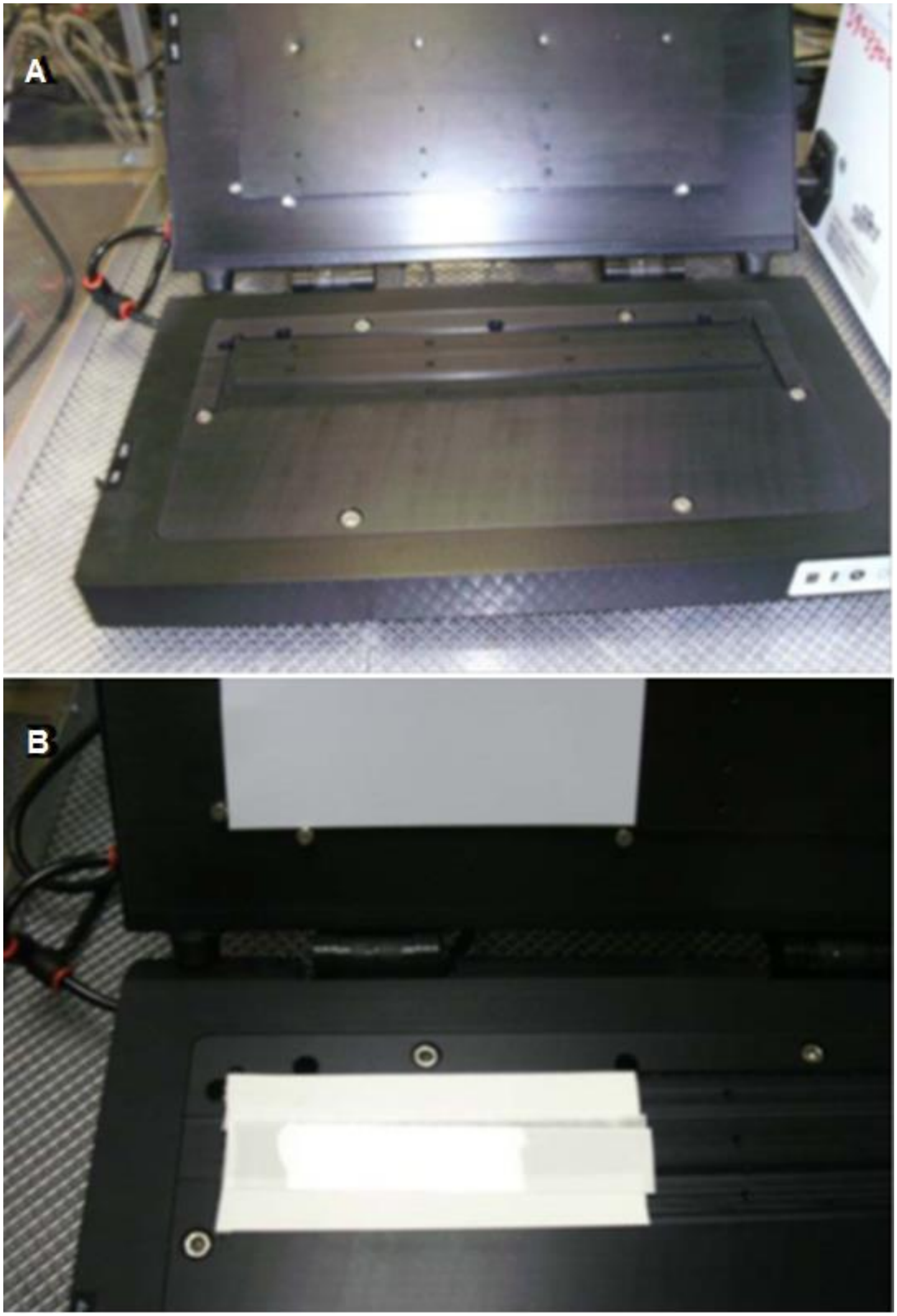

Figure 3.7: A. The batch laminating system, B. The membranes in the clamshell.

Figure 3.8: The guillotine cutting system.

3.2 Literature review

Immunochromatographic assays (lateral flow tests or simply strip tests), as one of the most useful and common biosensor application, combine chromatography with conventional immunoassay to offer a new analytical tool [59-60]. They are a logical extension of the technology used in latex agglutination tests, the first of which was developed in 1956 [61-62].The principle behind the test is straightforward. The first stage of immunochromatographic method is to use visible judgment (colorimetric) for the qualitative of the analyte. Any ligand that can be bound to a visually detectable solid support can be tested qualitatively, and in many cases even semi-quantitatively. Some of the more common lateral flow tests products currently on the market include the following: MySet™ for pregnancy (Ani Biotech Oy, Finland); NOW® Strep A Test for Strep throat (Binax, Inc. USA), and Onestep Chlamydia test for Chlamydia (Cortez Diagnostics, CA). A fluorescence immunochromatographic assay has also been developed for quantifying analytes in liquid samples [63], where Kim and et al. reported a cartridge-based test strip with two distinct fluorescent labeled indicators. The analyte of interest interacts with an indicator forming a fluorescent complex which is captured in the test zone while the second indicator remains intact within the test strip to provide an internal standard. In this regard, the PI has developed recently, in research collaboration with NDSU, a dry-reagent strip biosensor that enables qualitative (visual)/quantitative detection of protein within minutes [64].

Immunochromatographic assays are suitable tools to determine many kinds of biomarkers and early diagnose of diseases [65]. For examples, Prostate-specific antigen (PSA), the serum marker used for the detection of prostate cancer, was detected by CdSe and ZnS nanoparticle label immunochromatographic assay with the detection limit of 0.02 ng/ml [66-67]. More than that, Immunochromatographic assays can linked with electrochemical devices that provide a rapid, sensitive, and quantitative detection tools for the disease-related biomarkers in the blood [68].

Breast cancer is the most common type of cancer and one of the most common causes of cancer death among women [69]. Early detection is a major factor contributing to the 3.2 % annual decline in breast cancer death rates over the past 5 years [70, 71]. Many researches in biosensor were done to detect different kinds of protein and DNA segments produced in the blood by breast cancer [72-80] for the early diagnosis of the cancer. Carcinoembryonic antigen (CEA) as a tumor marker for many solid organ cancers [81] is considered as a good analyte to detect for early diagnosis of breast cancer. Many immunoassay methods, such as ELISA [82], radioimmunoassay (RIA) [83], fluorescence [84], chemiluminescence [85], and quartz crystal microbalance [86] were employed to separately detect CEA. Immunochromatographic assay was used in determine CEA marker [87].

Using nanoparticles as an enhanced factor for signal in addition to other benefits for the analytical process at all have developed immunochromatographic assays technique [88]. AuNPs as a good choice for nanoparticles to use for the technique can be synthesized according to the citrate reduction of $HAuCl_4$ [89] and used for the dry reagent strip biosensor to detect DNA by hybridization [90], label/immunochromatographic electrochemical biosensor (IEB) to detect prostate specific antigen (PSA) in human serum [91]. AuNPs probes for immunochromatographic strips to determine rabbit IgG and human IgM (H-IgM) [92], and to produce a disposable nucleic acid biosensor

(DNAB) for sensitive detection of nucleic acid samples [93]. AuNPs immunochromatographic strips were used to enhance the detection of CEA and achieve lower quantitative detection of the CEA marker [94-95].

On other hand, different kinds of targets like environmental analytes and biomarkers have been detected by using Enzyme-linked immune sorbent assay [96-97]. By apply different types of substrates and detected product, immunoglobulin M (IgM) antibodies were detected by enzyme-linked immunosorbent assay and protein-based enzyme-linked immunosorbent assay used for detection of IgG antibody [98-99]. Furthermore, Enzyme-linked immune sorbent with 4-chloro-1-naphthol as a substrate used to improve the point of care dry-reagent strip biosensors to detect IgG antibody [100].

3.3 Experimental section

3.3.1 Materials and methods

3.3.1.1 Reagents and membranes

Goat anti-rabbit IgG-alkaline phosphate conjugated, Rabbit IgG (whole molecule), and goat anti-rabbit IgG biotin were obtained all from Thermo scientific. Micro particles magnetic streptavidin coated was obtained from Floka. α-Naphthyl phosphate, α-nophthol, Tris-HCl, sodium hydroxide, hydrogen peroxide, sodium chloride, sodium phosphate, albumin from bovine serum were obtained from sigma. Supporting electrolytes (Phosphate buffer solution of different pHs) will be prepared from standard solutions of monosodium phosphate (NaH_2PO_4) and disodium phosphate (Na_2HPO_4). All solutions will be prepared with 18.2 MΏ deionized water.

cellulose ester membrane, glass fibers, non-woven cotton fiber material, and plastic Adhesive backing were obtained from Millipore, Chloroauric acid and trisodium citrate dehydrate were obtained from Sigma.

3.3.1.2 Apparatus and procedures

Lateral-flow immunochromatographic strip fabrication apparatus:

Bio Dot XYZ3050 system provided with three kinds of the dispensers: (1) The air jet dispenser (2) The frontline quanti dispenser (3) The biojet quanti dispenser, Bio Dot CM4000 guillotine cutting system, Bio Dot LM5000 batch laminating system, and D.T.1030 Kinbio tech Co test strip reader.

3.3.2 Procedures

3.3.2.1 The Gold Nanoparticles (AuNPs) preparation

The reducing agent is added, gold atoms are formed in the solution, and their concentration rises rapidly until the solution exceeds saturation. Particles form in a process called nucleation. The remaining dissolved gold atoms bind to the nucleation sites and growth occurs. However the Synthesize of AuNPs was done by following the next steps:

1. Add 250 mL of 1.0 mM Chloroauric acid ($HAuCl_4$) to a 500 mL beaker or Erlenmeyer flask on a stirring hot plate. Add a magnetic stir bar and bring the solution to a rolling boil

2. Keeping boiling for 15 min

3. Add 4.5 ml of a 1% solution of trisodium citrate dihydrate, $Na_3C_6H_5O_7.2H_2O$. The gold atoms gradually form as the citrate reduces the gold (III)

4. After 20s the color changes into dark blue

5. After 60s the color changes into wine-red

6. Stirring without heat for 15 min

3.3.2.2 Treatments of the membranes

Adsorption pad and the glass fiber (conjugate pad) are used directly without any pretreatment. On the other hand, the nitrocellulose membrane is blocked by using BSA the pretreatment is done by Dipping and soaking the membrane in buffer (PBS [phosphate buffered solution] pH 7.4 + 1% BSA [bovine serum albumen]) solution for 30 min then it is cleaned by dipping in double distilled water for washing the excess of solution and leave to dry at 37c for 40 min. To treat the Sample pad membrane, it is soaked in the following buffer (1.168 ml of 0.25% triton, 3.94 g of Tris HCl salt and 37.5 µl of NaCl 2 M for 500 ml) for ½ hr. then dried in the oven at 37c for 2hrs.

3.3.2.3 Antibody-AuNPs conjugate preparation

The Antibody-AuNPs conjugate preparation is achieved by following steps:

1. AuNPs (15 nm Au) solution is concentrated for 10 times in the centrifuge (15 min at 12 krpm) by using 10 vials 1 ml of the Au NPs solution. Then they are combined all in 1ml double distilled water vial.
2. The solution is adjusted to pH of 9.0 by using 4-5 µl of Na_2CO_3 0.2 M. The value of pH is tested by using pH paper (pH fix 0-14).
3. The goat anti-rabbit IgG is added to get 50 µg/ml of concentration of the antibody in the 1 ml and mixed gently for 1 hr at room temperature.

4. 100µl of 10% BSA is added to have a final BSA concentration 1% and block the remaining active sites of AuNPs then mixed gently for ½ hr at room temperature.
5. Antibody-AuNPs conjugated is washed by following:

   The solution is centrifuged for 15min at 12 Krpm rate, then the liquate is removed, 1 ml of (PBS + 1% BSA) is added by using the pipette. Washing is repeated 3 times in the same steps.

6. The final solution is dispensed in 1 ml of buffer (0.076g of $Na_3PO_4.12H_2O$ 20 mM, 1.0g of sucrose 10%, 0.5 g of BSA 5% and 22.7 µl of tween-20 0.25% for 10 ml)

3.3.2.4 Fabrication and test of the lateral flow strip

Goat anti-rabbit IgG is diluted to 1.15 mg/ml by using phosphate buffer solution pH of 7.4 and dispensed on the test zone by biojet quanti dispenser. After the fabrication of the strip as mentioned in the introduction (Figure 3.9), antibody-AuNPs conjugate solution is applied on the conjugate pad using the pipette. At this level the strip is ready to test. Rabbit IgG antigen is diluted to 100 µg/ml using BPS in different concentrations. 100 µl of the sample of rabbit IgG is applied in the sample pad and after 2 minutes 50 µl of running buffer (PBS+ Tween+ BSA) is applied in the sample pad. The measurement is taken and recorded after 10 minutes using the strip reader (Figure 3.10).

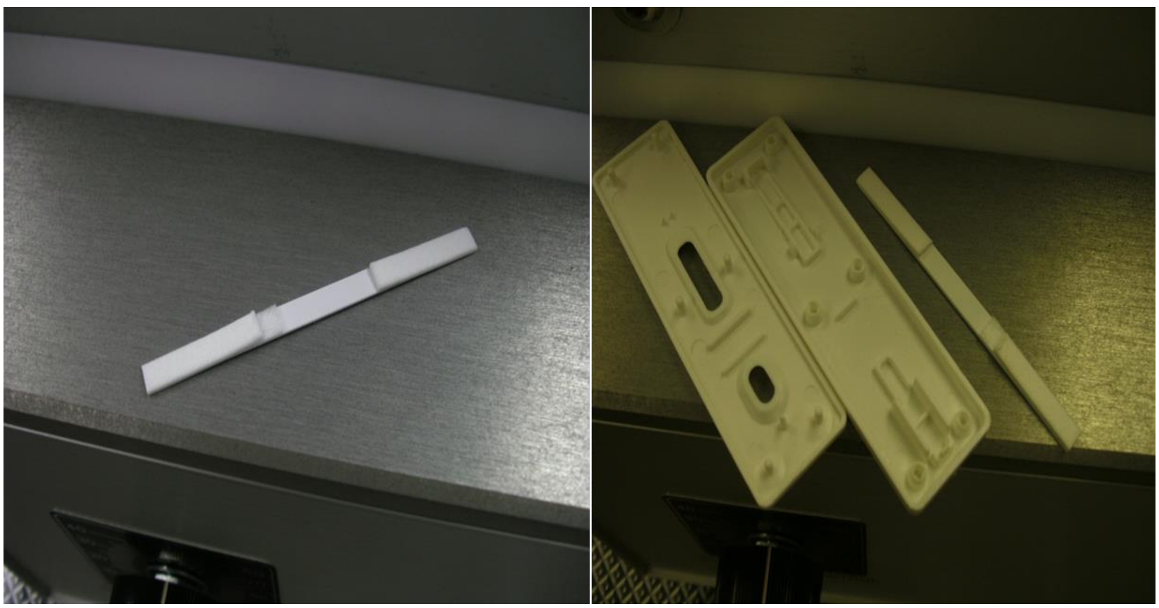

Figure 3.9: The lateral flow strip.

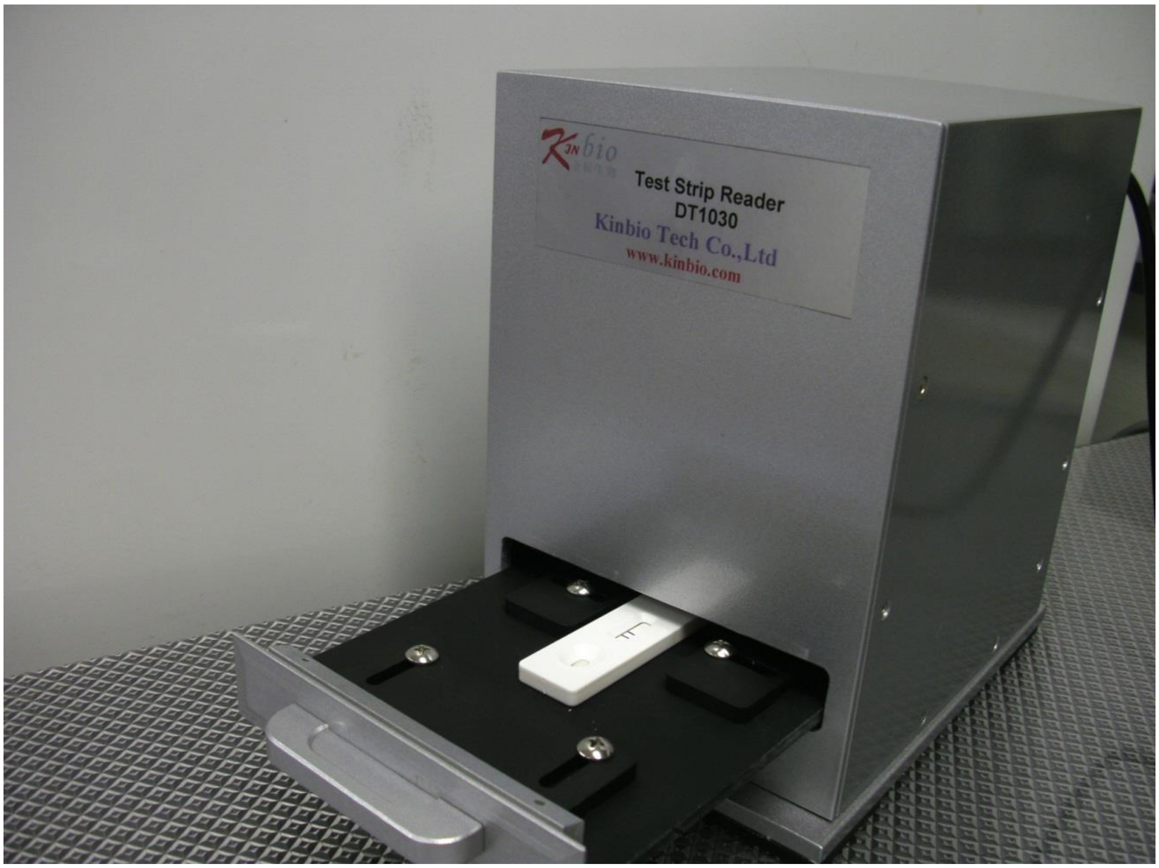

Figure 3.10: The test strip reader.

3.4 Results and discussion

3.4.1 AuNPs U.V Characterization

The spectroscopy measurement was done for Au NPs solution Synthesized and the peak was given at 523.97 nm wavelength (Figure 3.11). The result was made sure that the synthesize process is achieved and formed AuNPs in the size of 15 n.m.

3.4.2 Optimization

3.4.2.1 Effect of nitrocellulose membrane sources

To study the effect of nitrocellulose membrane type on the test operation, six types of membranes are study including of HF 75, HF 90, HF 120, HF 135, HF 180, HF 240 (Table 3.1). The difference between these membranes is the capillary flow time due to the size of porous in the chemical structure (Figure 3.12). As noticed, the smaller porous size gives slower flow rate leading to lower capillary flow time. To optimize the type of nitrocellulose membrane, photo images and response signals of the test strip with a portable strip reader are recorded (Figure 3.13). The signal to noise ratio is calculated for the six tests and as we noticed the HF 180 nitrocellulose membrane gives as a best result with 137 of ratio (Figure 3.14). As it is clear, the size of the porous and structure of nitrocellulose membrane play a role in the set the goat anti-rabbit IgG and their fixed number on the test zone, because of that the number of antigen goat anti-rabbit IgG complex captured on the test zone in addition to change the rate of flow in the structure of membrane for the different components of sample applied in the sample pad.

Table 3.1: Nitrocellulose membrane types

Membrane	Capillary Flow Time (sec/4 cm)
HF (Hi-Flow) 75	75
HF 90	90
HF 120	120
HF 135	135
HF 180	180
HF 240	240

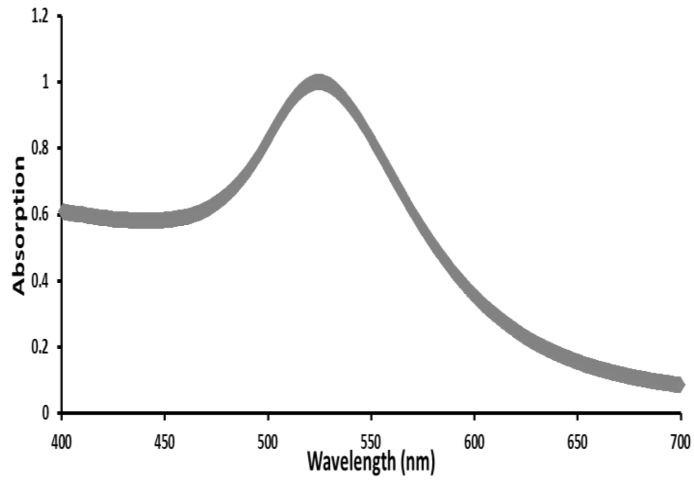

Figure 3.11: The spectroscopy measurement of the 15nm gold nanoparticles.

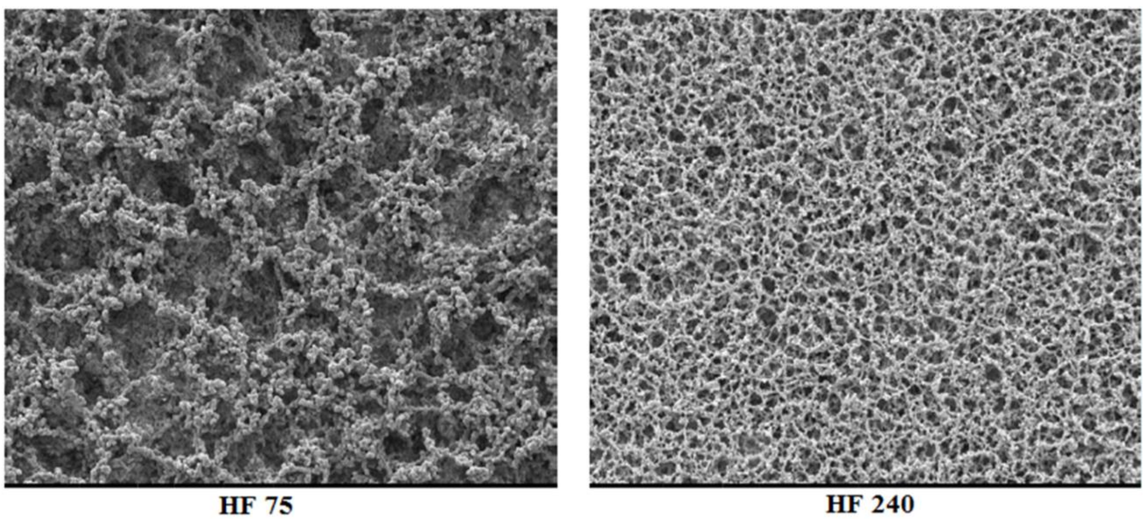

Figure 3.12: photo shows the structure of two types of nitrocellulose membranes.

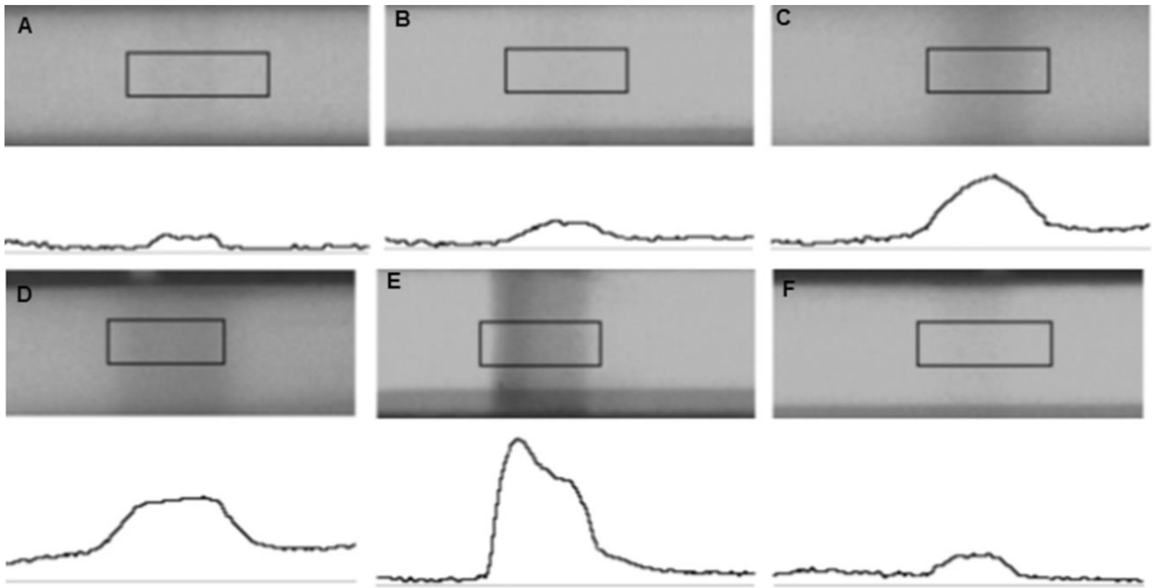

Figure 3.13: Typical photo images and recorded response signals of the test strip with a portable strip reader after applying the sample solutions. (A) HF 75, (B) HF 90, (C) HF 120, (D) HF 135, (E) HF 180, (F) HF 240 nitrocellulose membrane. All sample solutions were prepared with 0.25% triton + Tris HCl + NaCl 2 M buffer; phosphate buffer pH 7.4 with tween + 1% BSA solution as a running buffer; 2 µl of Au-anti-Rabbit IgG conjugates on conjugates pad; 3 times of dispersing of goat anti-rabbit IgG of 1.15 mg/ml prepared in phosphate buffer solution pH 7.4 on test line; cellulose fiber sample pad type S 2000 with 2 cm length.

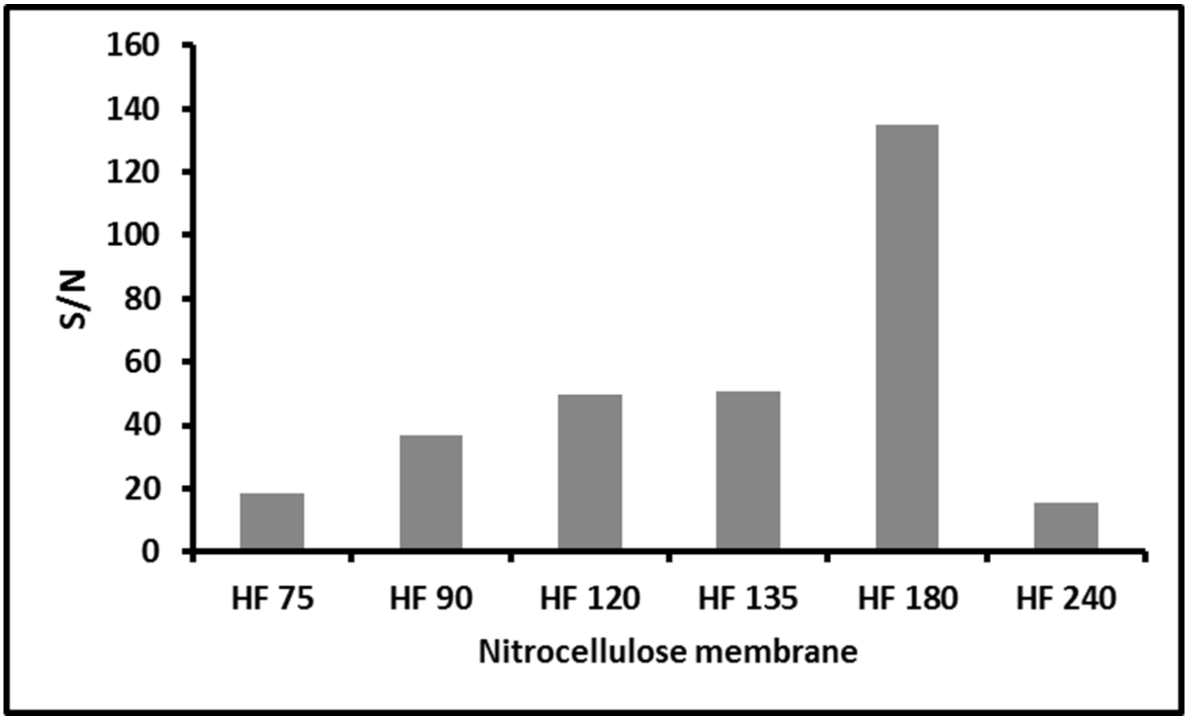

Figure 3.14: Effect of nitrocellulose membrane sources on the signal to noise ratio of the biosensor. All sample solutions were prepared with 0.25% triton + Tris HCl + NaCl 2 M buffer; phosphate buffer pH 7.4 with tween + 1% BSA solution as a running buffer; 2 µl of Au-anti-Rabbit IgG conjugates on conjugates pad; 3 times of dispersing of goat anti-rabbit IgG of 1.15 mg/ml prepared in phosphate buffer solution pH 7.4 on test line; cellulose fiber sample pad type S 2000 with 2 cm length.

3.4.2.2 Effect of cellulose fiber sample pad length

Two types of cellulose fiber sample pad studied in our research. The difference in the two types is just in the length of the pad applied in the lateral strip so we have two length of pad applied 1.7 and 2.0 cm. photo images and recorded response signals are shown in (Figure 3.15). From the signal to noise ratio figure, the ratio of 1.7 cellulose fiber sample pad type was a slightly higher than which belongs to 2.0 type. According to this result, the type of 1.7 cm cellulose fiber sample pad is chosen as an optimized type (Figure 3.16). As expected explanation for the result, the length of the pad may play a role in prevent a tiny part of analyte to move into the conjugate pad and captured after that on the test zone or we can say that the contact area between the sample pad and conjugate pad play a role in moving the analyte and the components of sample. However, the difference between the lengths of sample pad has a little effect on the signal to noise ratio so on the measurement process in general.

3.4.2.3 Effect of BSA% in the sample buffer

Three different percent of BSA in addition of case of absence the material were studied. The photo images and recorded response signals of the test strip for the four tests are shown in (Figure 3.17). From the signal to noise ratio calculated, it was clearly that the ratio increases proportional to decreasing the BSA%, the highest ratio recorded in the case of absence of BSA in the sample solution and chosen as an optimized condition for the following tests (Figure 3.18).

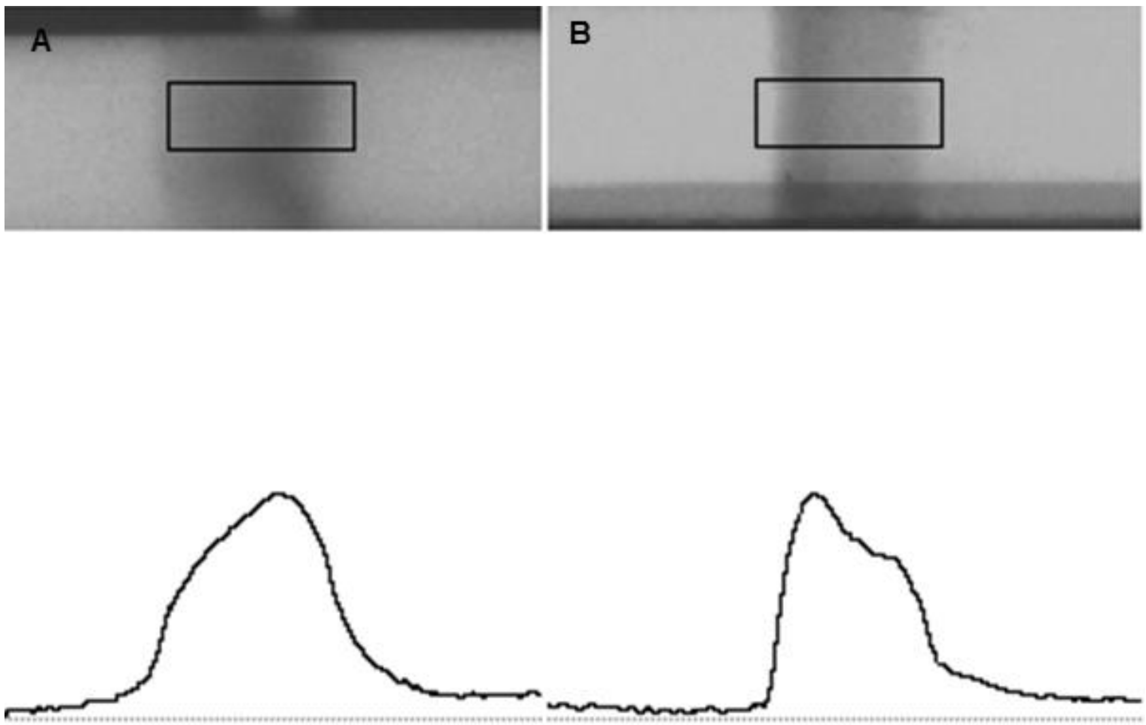

Figure 3.15: Typical photo images and recorded response signals of the test strip with a portable strip reader after applying the sample solutions. (A) 1.7 cm, (B) 2.0 cm cellulose fiber sample pad length. All sample solutions were prepared with 0.25% triton + Tris HCl + NaCl 2 M buffer; phosphate buffer pH 7.4 with tween + 1% BSA solution as a running buffer; 2 µl of Au-anti-rabbit IgG conjugates on conjugates pad; 3 times of dispersing of goat anti-rabbit IgG of 1.15 mg/ml prepared in phosphate buffer solution pH 7.4 on test line; nitrocellulose membrane HF 180.

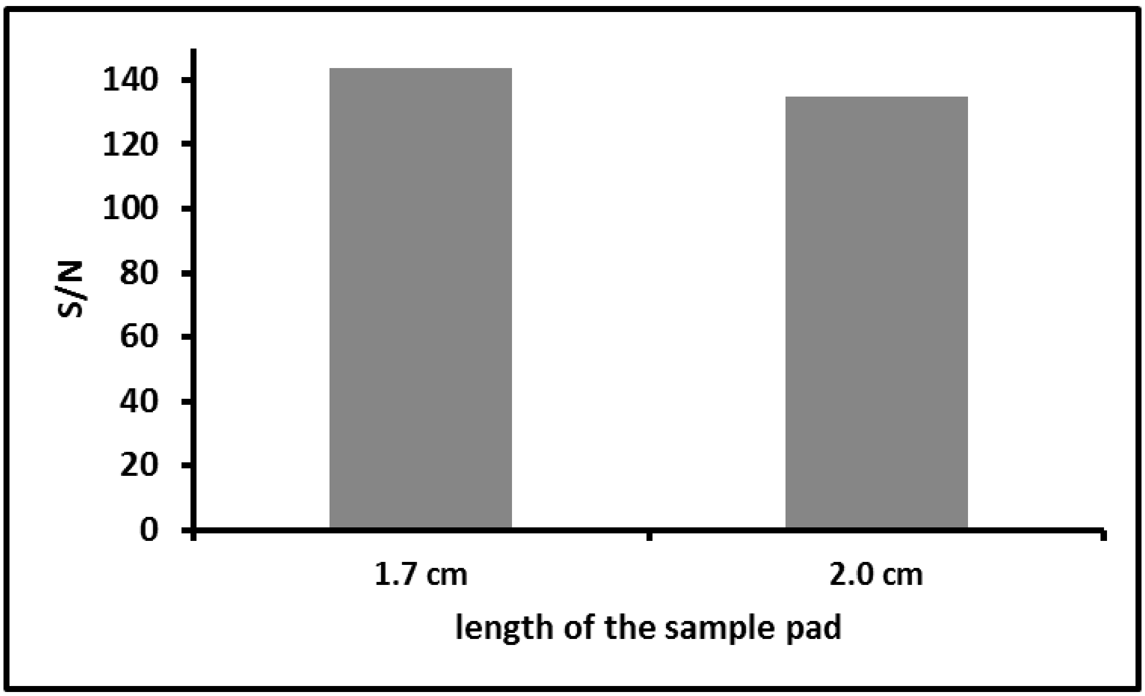

Figure 3.16: Effect of cellulose fiber sample pad length on the signal to noise ratio of the biosensor. All sample solutions were prepared with 0.25% triton + Tris HCl + NaCl 2 M buffer; phosphate buffer pH 7.4 with tween + 1% BSA solution as a running buffer; 2 µl of Au-anti-Rabbit IgG conjugates on conjugates pad; 3 times of dispersing of goat anti-rabbit IgG of 1.15 mg/ml prepared in phosphate buffer solution pH 7.4 on test line; nitrocellulose membrane HF 180.

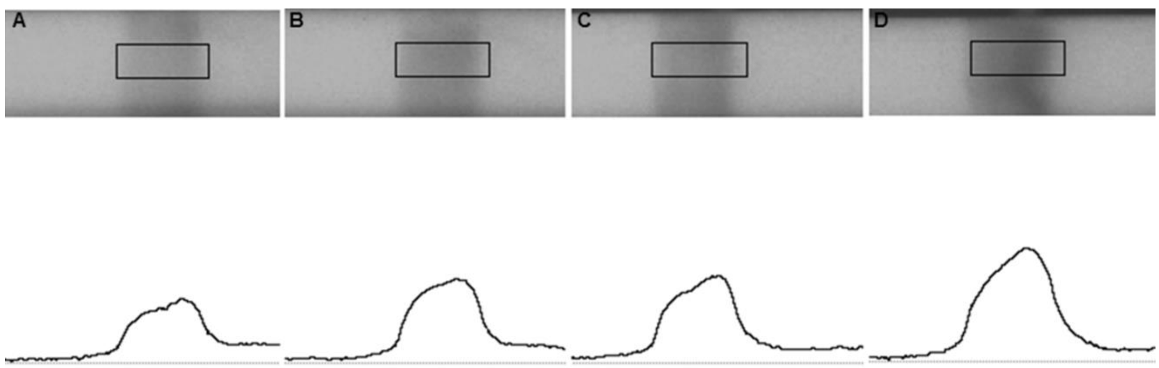

Figure 3.17: Typical photo images and recorded response signals of the test strip with a portable strip reader after applying the sample solutions. (A) 3%, (B) 2%, (C) 1%, (D) 0% of BSA in the sample buffer. All sample solutions were prepared with 0.25% triton + Tris HCl + NaCl 2 M buffer; phosphate buffer pH 7.4 with tween + 1% BSA solution as a running buffer; 2 µl of Au-anti-rabbit IgG conjugates on conjugates pad; 3 times of dispersing of goat anti-rabbit IgG of 1.15 mg/ml prepared in phosphate buffer solution pH 7.4 on test line; nitrocellulose membrane HF 180.

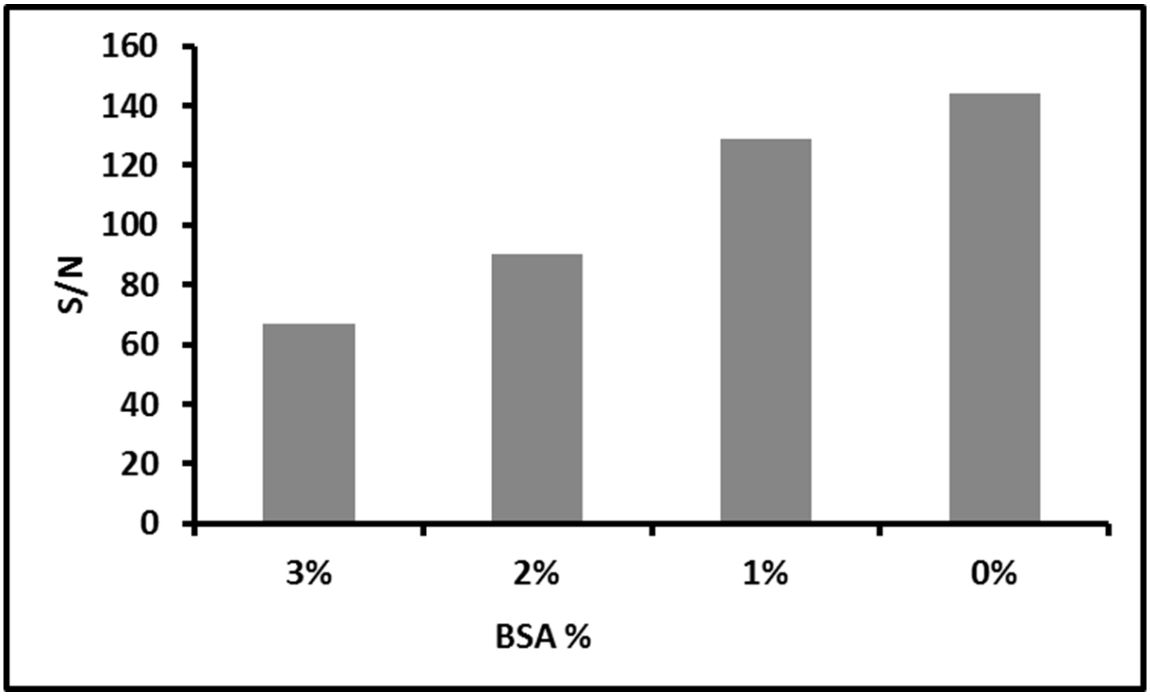

Figure 3.18: Effect of BSA% in the sample buffer on the signal to noise ratio of the biosensor. All sample solutions were prepared with 0.25% triton + Tris HCl + NaCl 2 M buffer; phosphate buffer pH 7.4 with tween + 1% BSA solution as a running buffer; 2 µl of Au-anti-rabbit IgG conjugates on conjugates pad; 3 times of dispersing of goat anti-rabbit IgG of 1.15 mg/ml prepared in phosphate buffer solution pH 7.4 on test line; nitrocellulose membrane HF 180.

3.4.2.4 Effect of tween% in the sample buffer

Study of effect of tween% in the sample buffer on the signal and noise covered three concentrations of tween 0.5%, 0.1% and 0% and recorded as typical photos and response signal (Figure 3.19). The experiments showed that the signal to noise ratio was at the highest recorded value in the case of absence of tween while the presence of tween affect the ratio negatively (Figure 3.20).

3.4.2.5 Effect of dispensing time of goat anti-rabbit IgG

It was noticed from the results that increasing of the dispensing of time on the test zone causes increase of the goat anti-rabbit IgG molecules on the zone which leads to capture more anlayte-anti-body-AuNPs conjugate molecules on the test line (Figure 3.21). The signal increases proportional to rise of dispensing time, however at the four dispensing time the signal to noise ratio is recorded as the highest, and remained constant for the five dispensing time (Figure 3.22).

3.4.2.6 Effect of Antibody-AuNPs volume

In general the signal increases by increasing antibody-AuNPs volume on the conjugate pad the number of anlayte-anti-body-AuNPs conjugate molecules increases. These molecules are captured on the test zone increasing the signal recorded (Figure 3.23). The signal to noise ratio increases gradually by increasing the antibody-AuNPs volume and reaches a peak at 5 µl, then the ratio goes down due to increasing of noise (Figure 3.24).

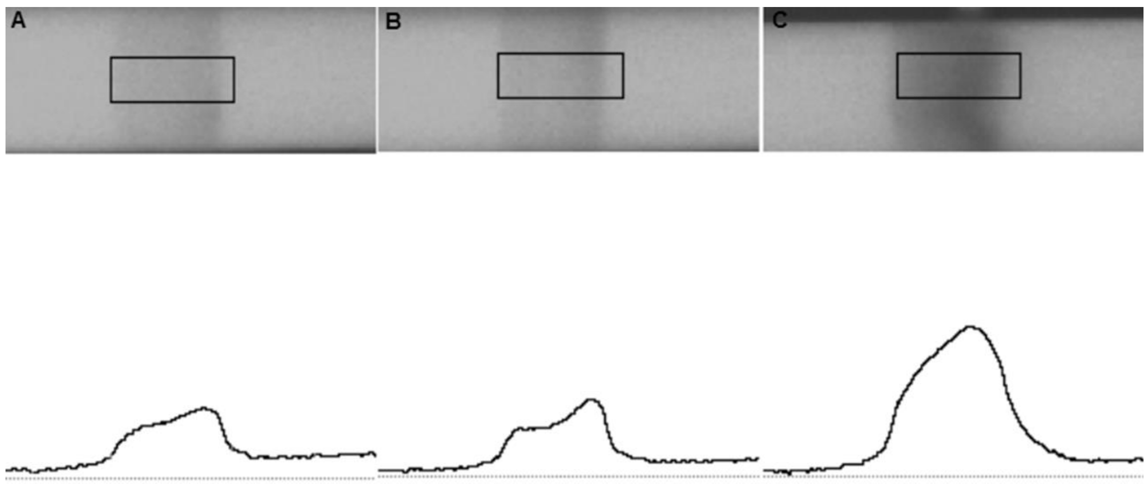

Figure 3.19: Typical photo images and recorded response signals of the test strip with a portable strip reader after applying the sample solutions. 0.50%, (B) 0.10%, (C) 0% of tween in the sample buffer. All sample solutions were prepared with 0.25% triton + Tris HCl + NaCl 2 M buffer; phosphate buffer pH 7.4 with tween + 1% BSA solution as a running buffer; 2 µl of Au-anti-Rabbit IgG conjugates on conjugates pad ; 3 times of dispersing of goat anti-rabbit IgG of 1.15 mg/ml prepared in phosphate buffer solution pH 7.4 on test line; nitrocellulose membrane HF 180; cellulose fiber sample pad type S 1700 with 1.7 cm length.

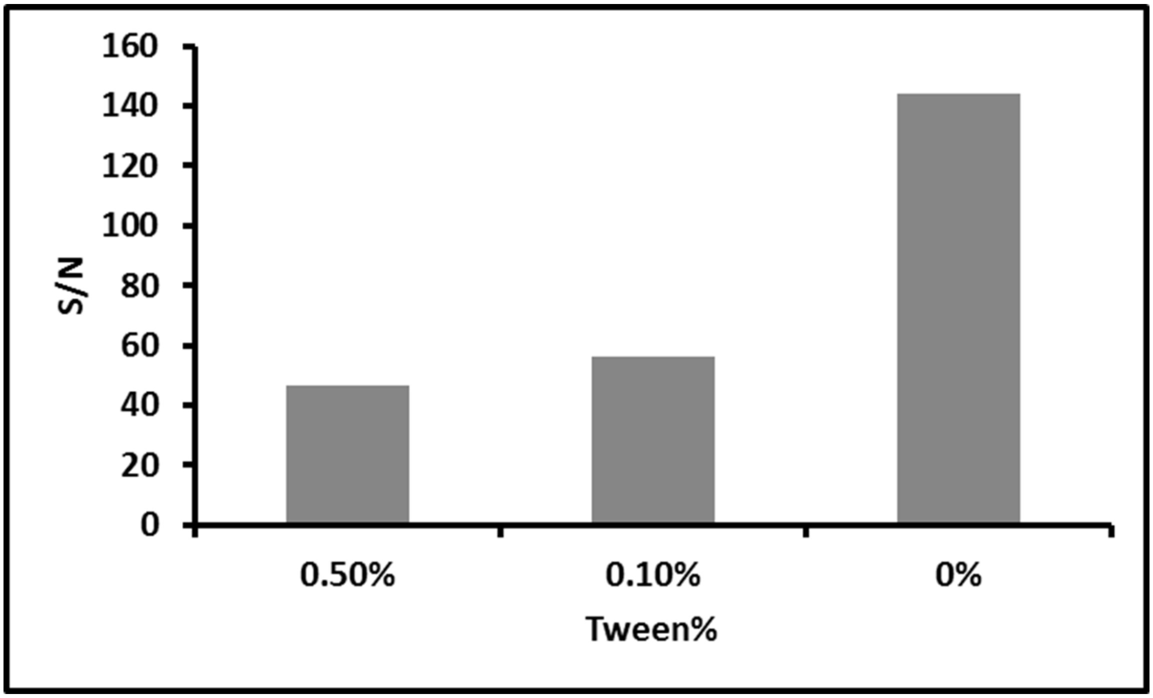

Figure 3.20: Effect of tween% in the sample buffer on the signal to noise ratio of the biosensor. All sample solutions were prepared with 0.25% triton + Tris HCl + NaCl 2 M buffer; phosphate buffer pH 7.4 with tween + 1% BSA solution as a running buffer; 2 µl of Au-anti-rabbit IgG conjugates on conjugates pad; 3 times of dispersing of goat anti-rabbit IgG of 1.15 mg/ml prepared in phosphate buffer solution pH 7.4 on test line; nitrocellulose membrane HF 180; cellulose fiber sample pad type S 1700 with 1.7 cm length.

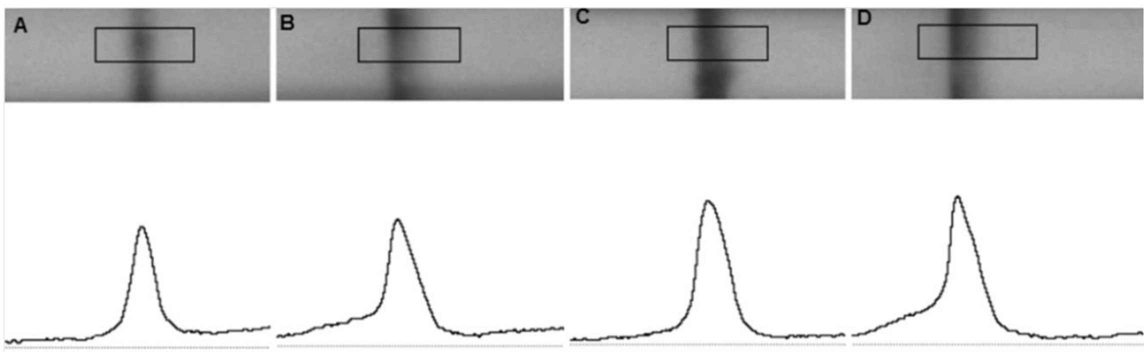

Figure 3.21: Typical photo images and recorded response signals of the test strip with a portable strip reader after applying the sample solutions. (A) 2, (B) 3, (C) 4, (D) 5 dispensing time. All sample solutions were prepared with 0.25% triton + Tris HCl + NaCl 2 M buffer; phosphate buffer pH 7.4 with tween + 1% BSA solution as a running buffer; 2 µl of Au-anti-rabbit IgG conjugates on conjugates pad; nitrocellulose membrane HF 180; cellulose fiber sample pad type S 1700 with 1.7 cm length.

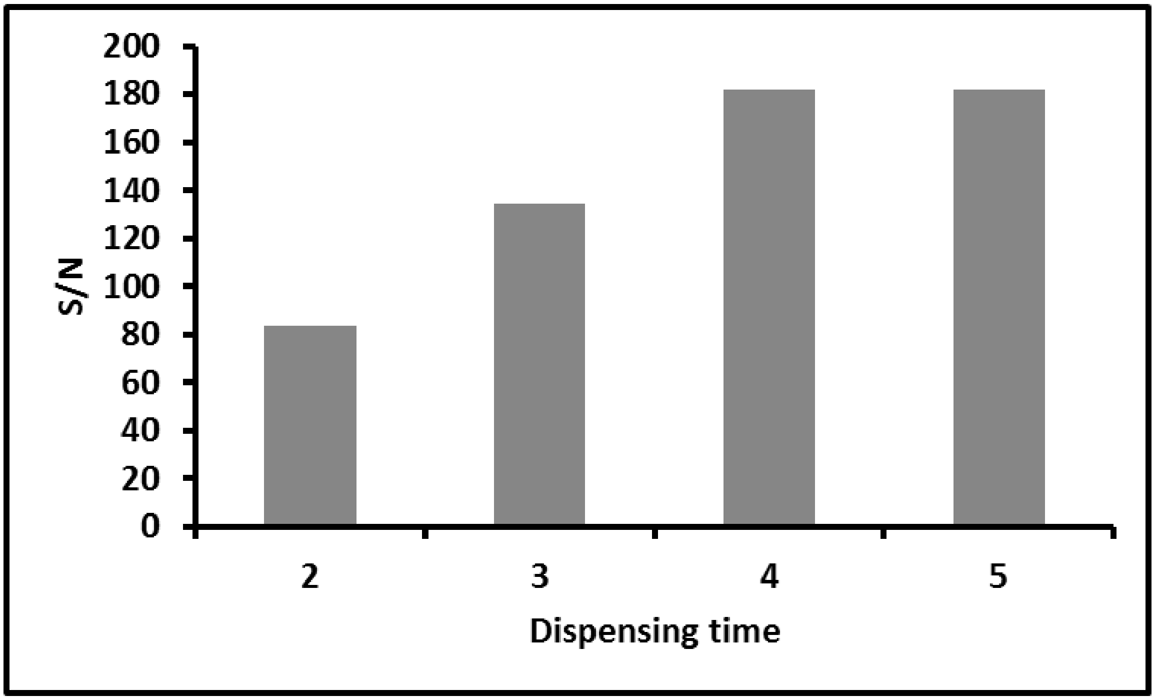

Figure 3.22: Effect of dispensing time on the signal to noise ratio of the biosensor. All sample solutions were prepared with 0.25% triton + Tris HCl + NaCl 2 M buffer; phosphate buffer pH 7.4 with tween + 1% BSA solution as a running buffer; 2 µl of Au-anti-rabbit IgG conjugates on conjugates pad; nitrocellulose membrane HF 180; cellulose fiber sample pad type S 1700 with 1.7 cm length.

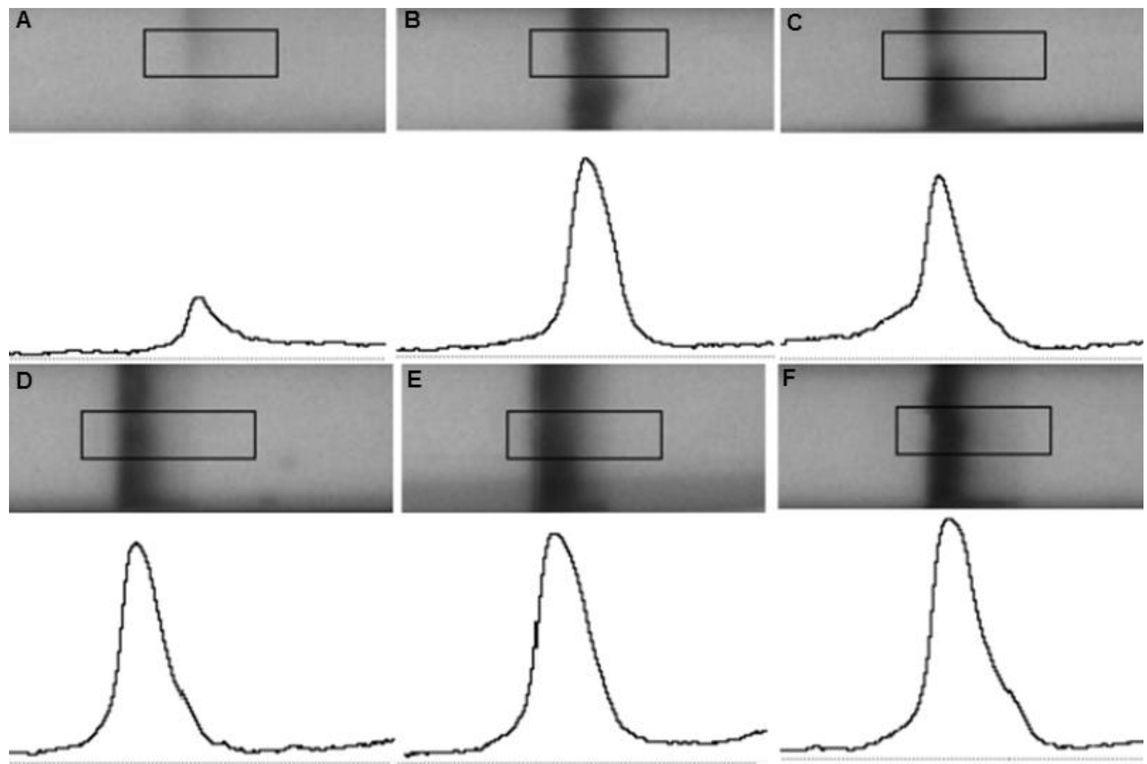

Figure 3.23: Typical photo images and recorded response signals of the test strip with a portable strip reader after applying the sample solutions. (A) 1, (B) 2, (C) 3, (D) 4, (E) 5, (F) 6 µl of Gold nanoparticles. All sample solutions were prepared with 0.25% triton + Tris HCl + NaCl 2 M buffer; phosphate buffer pH 7.4 with tween + 1% BSA solution as a running buffer; 4 times of dispersing of goat anti-rabbit IgG of 1.15 mg/ml prepared in phosphate buffer solution pH 7.4 on test line; nitrocellulose membrane HF 180; cellulose fiber sample pad type S 1700 with 1.7 cm length.

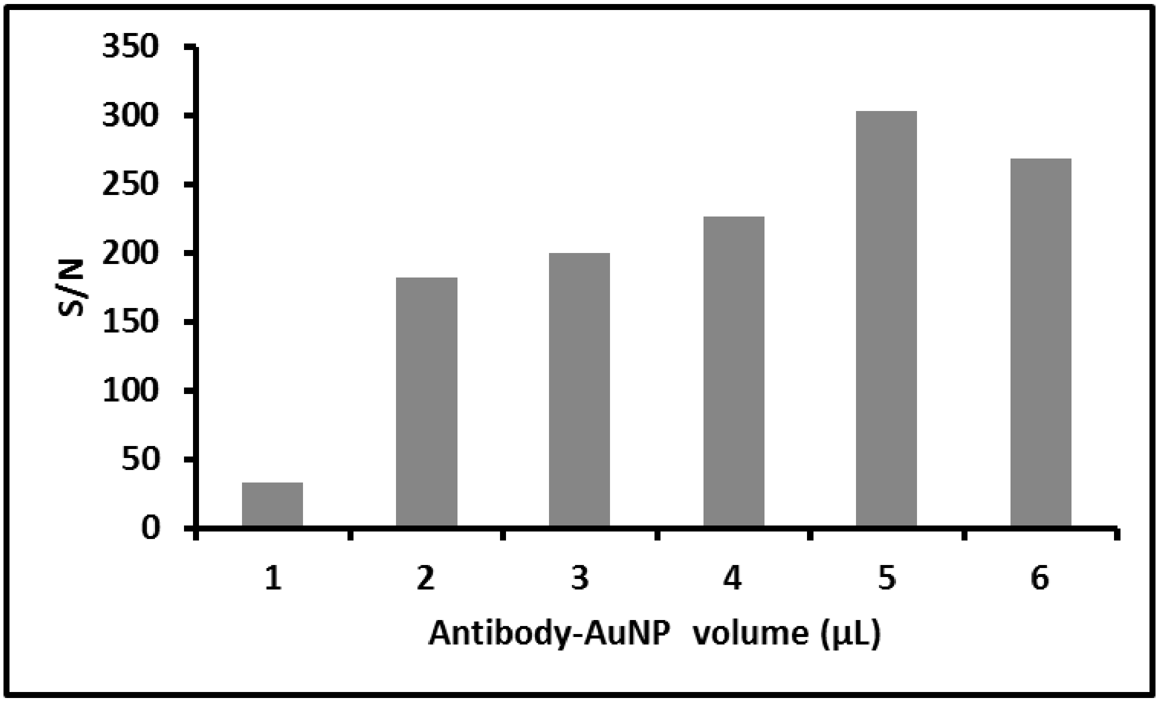

Figure 3.24: Effect of Antibody-AuNP volume on the signal to noise ratio of the biosensor. All sample solutions were prepared with 0.25% triton + Tris HCl + NaCl 2 M buffer; phosphate buffer pH 7.4 with tween + 1% BSA solution as running buffer; 4 times of dispersing of goat anti-rabbit IgG of 1.15 mg/ml prepared in phosphate buffer solution pH 7.4 on test line; nitrocellulose membrane HF 180; cellulose fiber sample pad type S 1700 with 1.7 cm length.

3.4.2.7 Detection of rabbit IgG antigen using lateral flow test

The calibration curve of α-naphthol was plotted by testing different concentrations of rabbit IgG (Figure 3.25). The equation form adapted in the calibration of the inset of (Figure 3.26) is S/N = 13.716 log [Rabbit IgG] ng/ml + 5.8195 and R^2 = 0.9866. The limit of quantification of the developed sensor is 1 ng/ml and the detection limit (at 3σ) is 0.48 ng/ml.

The reproducibility of the sensor was studied by testing replicate measurements and the relative standard deviation was determined 4.4%.

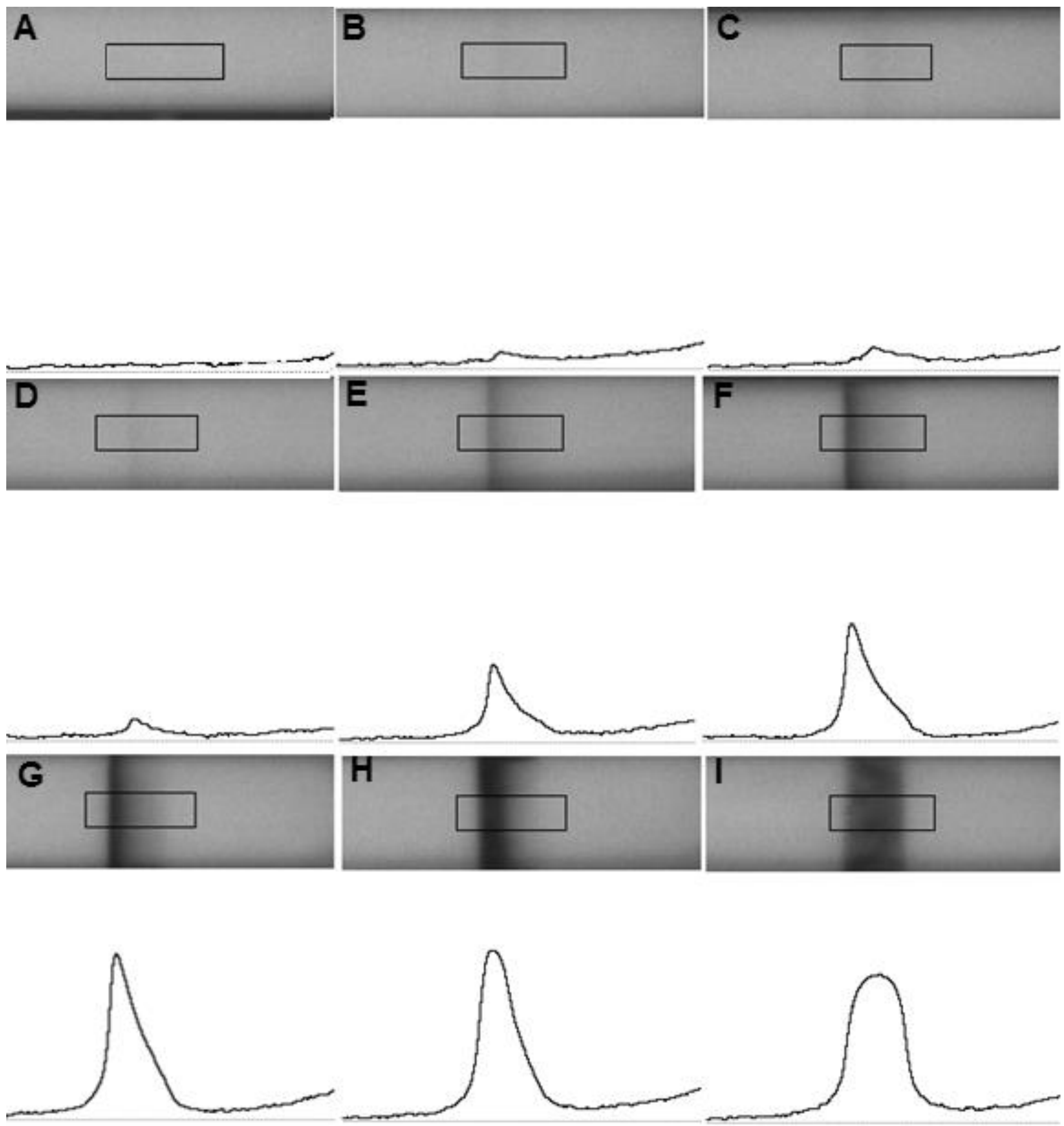

Figure 3.25: Typical photo images and recorded response signals of the test strip with a portable strip reader after applying the sample solutions. (A) Blank, (B) 1, (C) 10, (D) 50, (E) 100, (F) 500, (G) 1000, (H) 10000, (I) 50000 ng/ml of Rabbit IgG.

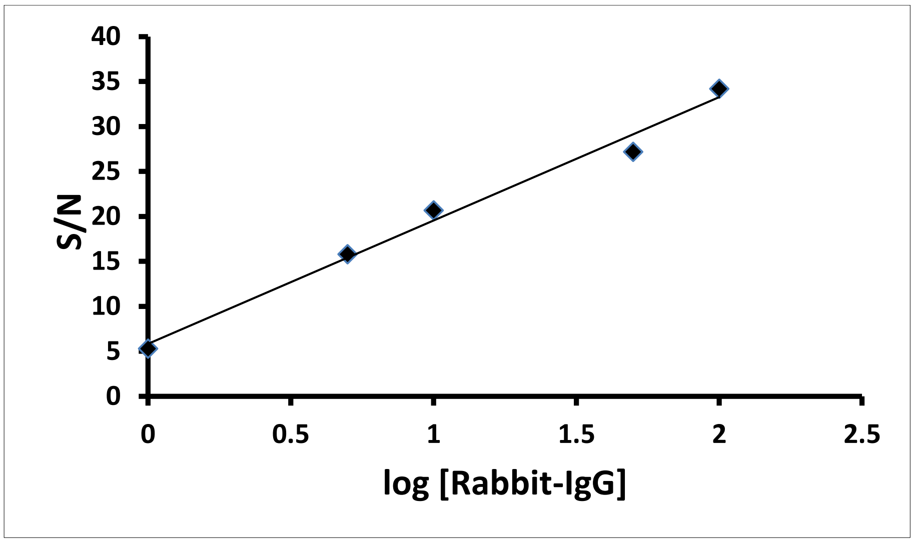

Figure 3.26: Representation of the corresponding calibration plot.

3.5 Conclusion

Lateral flow immunochromatographic assays are an active field in the biosensor area and medicine industry, this interesting due to the benefits of immunochromatographic tests like short time of test result, less interference, long-term stability, inexpensive manufacture and user-friendly format make from. Our research focuses on the design of lateral flow test to detect rabbit IgG antigen. The fabrication was achieved starting with synthesize of AuNPs (size of 15 n.m) and characterization was done by the spectroscopy measurement at 523nm wavelength. To fabricate the lateral flow strip, the following step started with making a pretreatment for the sample pad and nitrocellulose membranes. Then the nitrocellulose membrane was prepared by dispensing the goat anti-rabbit IgG on the test zone using the biojet chemical system. After that, assembling of the lateral flow assay comprised of materials on the order of a nitrocellulose membrane, a sample pad, a conjugate pad, and an absorbent pad was done by using batch laminating system. The final size and length of the strip is designed and produced using the guillotine cutting system. At the end, AuNPs goat anti-rabbit IgG conjugate solution was applied on the conjugate pad, the sample contained a certain concentration of rabbit IgG was applied in the sample pad, after two minutes a washing buffer was applied on the sample pad. To record the signal appeared on the test line the strip was put in the strip portable reader and run the instrument.

To develop the performance of the lateral flow produced strip, the optimization were done over many components and solutions used in the preparation including run solution, percent of tween and BSA in the run solution, the volume of AuNPs goat anti-rabbit IgG conjugates was applied on the conjugate pad, time of dispersing of goat anti-rabbit IgG on the test zone, the type of nitrocellulose membrane and the size of the cellulose fiber sample pad type. After parameters optimization, the sample solutions were prepared with 0.25% triton + Tris HCl + NaCl 2 M buffer, phosphate buffer pH 7.4 with tween + 1% BSA solution as a running buffer, 5 µl of Au-anti-rabbit IgG conjugates on conjugates pad, 4 times of goat anti-rabbit IgG of 1.15 mg/ml prepared in phosphate buffer solution pH 7.4 on test line, nitrocellulose membrane HF 180, cellulose fiber sample pad type S 1700 with 1.7 cm length. The relative standard deviation (RSD) was determined as 4.4 %. The limit of quantification of developed sensor is 1 ng/ml; the detection limit (at 3σ) is estimated to be 0.48 ng/ml.

CHAPTER 4

Summary and future prospective

4.1 Summary

Immunoassay is a major application of the biosensor, the immunsensor is used to detect or quantify a specific substance, the analyte, in a sample, using an immunological reaction. The current research focuses on two types of immunoassays which are enzymatic immunoassay based on the magnetic beads and strip based dry immunoassay. The first type is explained in the chapter two, this part of the research explores the design and fabrication of enzymatic immunosensor based on the magnetic beads to detect rabbit IgG antigen as a model for the biosensor.

Streptavidin-coated magnetic beads are reacted with biotinylated anti-rabbit IgG. Then a sample contained rabbit IgG target can be added, followed with alkaline phosphatase conjugated anti-rabbit IgG adding to form the sandwich compound. α-Naphthyl phosphate is used as a substrate and reacts on the phosphatase enzyme surface and produce α–naphthol as a product which can be detected using electrochemical transducer. the electrochemical transducer in based on the three electrodes system using the Ag/AgCl as reference electrode , platinum wire counter electrode and graphite pencil electrode selected among four different carbon electrodes which were tasted for the selection.

The testing of biosensor is depended on the study of signal to noise ratio. The effect of different concentrations of components are studied and optimized and as the result, the final volume and concentration depended are the magnetic beads amount is 25 µg, the concentration of the biotinylated anti-Rabbit IgG is 100 µg/ml and the concentration of the alkaline phosphatase conjugated anti-Rabbit IgG is 10 µg/ml. To enhance the signal produced by square wave voltammetry on the surface of the graphite pencil electrode, all the effect parameters were optimized including potential increment, amplitude, frequency and accumulation potential. The optimized parameters adopted in our work are 0.015 V increment potential, 0.1 V amplitude, 30 Hz frequency and without applying accumulation potential.

The second type of immunosensor studied in chapter three. The study includes the fabrication and design a lateral-flow chromatographic strip that can be used for various sensing and analytical applications. In our research we use rabbit-IgG as a model and the fabrication is done following certain steps. The fabrication process starts with pretreatment of Membranes used and the goat anti-rabbit IgG is dispensed on the test zone of the nitrocellulose membrane using the biojet chemical system. After that, assembling of the lateral flow assay comprised of materials on the order of a nitrocellulose membrane, a sample pad, a conjugate pad, and an absorbent pad is done by using batch laminating system. After the assembling step, the guillotine cutting system is used to produce the last form of the strip with the length and size required.

AuNPs goat anti-rabbit IgG conjugate solution is dispensed on the conjugate pad by pipette and rabbit-IgG sample is applied in the sample pad, after two minutes a washing buffer is applied on the sample pad. To record the signal appeared on the test line the strip is put in the strip portable reader and run the instrument.

The optimization was done over many components and solutions. According to the optimization, The sample solutions were prepared with 0.25% triton + Tris HCl + NaCl

(2M) buffer, phosphate buffer pH 7.4 with tween + 1% BSA solution as a running buffer, 5 μl of Au-anti-Rabbit IgG conjugates on conjugates pad, 4 times of goat anti-Rabbit IgG of 1.15 mg/ml prepared in phosphate buffer solution pH 7.4 on test line, nitrocellulose membrane HF 180, cellulose fiber sample pad type S 1700 with 1.7 cm length.

As comparison between the two achieved biosensors, several advantages of the lateral-flow chromatographic strip design have been noticed. Firstly, the detection time is relatively short, where 10 min are required to record the result after applying the sample. Secondly, it is possible to detect the presence or the absence of the target in the matrix. Lastly, the biosensor does not need any chemical adding or specialist to run the detection process. These benefits make the fabricated biosensor a proposed and simple tool which can be used in the process of protein detection in general and domestic breast cancer diagnosis. On the other hand, the magnetic beads based electrochemical biosensor requires 120 min to record the result after applying the sample. In addition, it needs specialist to run the electrochemical detection processes. Both biosensors recorded the same limit of quantification and slightly no huge difference between the estimated limits of detection.

4.2 Future prospective

Starting with the magnetic beads based enzymatic immunosensor; the developing of the sensor is can be achieved on many levels. As we use alkaline phosphatase enzyme bioreceptor (molecular recognizer), the ability of using other kind of enzymes is one of the proposed mechanisms to develop the biosensor. In this way we can apply other kind of substrate belongs to enzyme used, thus different kinds of products can be formed and detected on the surface on the electrodes with other ability for lower LOD, LOQ and better signal recorded. However, some products can be color, and determined by optical transducers. For examples, using the Horseradish peroxidase (HRP) conjugated with anti-IgG and applied the substrate of 4-chloro-1-naphthol and H_2O_2. After the enzymatic reaction, we can get a colorimetric measurable blue product. On other side, we can develop the transducer part by develop the sensor. Developing the electrochemical sensor can be done by testing other kinds of working electrode. Another way to develop the sensor can be experimented by develop the graphite pencil electrode itself which enhances the signal.in other words, modifying the electrode by using different kind of nanoparticles like palladium and gold nanoparticles is new methods followed to enhance signal produced.

Moving to lateral flow immunochromatographic assays part, the developing of biosensor can be tested by practicing different kind of nanoparticles like palladium and silver nanoparticles. Second way can proposed in this area is changing the design of the lateral flow strip. The order of membrane mentioned in chapter three can be considered as a standard design of the lateral flow strip. However, another type of design order of the membranes and size of membranes applied can play a role in the mechanism of detection and the signal produced. On other side, we used in our research the optical transducer to detect the signal. Electrochemical transducer is also an able applied instrument in the biosensor to transduce the reaction of the electrode's surface of nanoparticles or product enzymatic formed to electric signal. In general, the electrochemical transducer can be applied by making a touch between the test zone and the electrodes surfaces of a special design assembling electrodes plate like screen printed electrodes.

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