T.R.
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF SCIENCE FACULITY
DEPARTMENT OF BIOLOGY

CHARACTERIZATION OF HRP-BASED AMPEROMETRIC BIOSENSOR BY USING SCREEN PRINTED ELECTRODES

Prepared By
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Supervisor
Assist. Prof. Dr. Ebru SAATÇİ

M. Sc. Thesis

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KAYSERI

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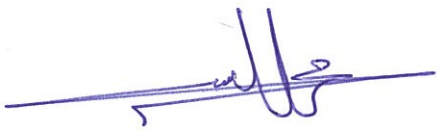
SCIENTIFIC ETHICS SUITABILITY

I declare that all information in this work was obtained in accordance with academic and ethical rules. All results and materials that not been at the essence of this work are also transferred and expressed by giving reference as required by these rules and behavior.



Mahmood Taha Noori AL-SADOON

SUITABILITY FOR GUIDE

The M.Sc. thesis entitled **"Characterization of HRP-based Amperometric Biosensor by Using Screen printed electrodes"** has been prepared in accordance with Erciyes University School of Natural and Applied Sciences Thesis Preparation and Writing Guide.

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ACCEPTANCE AND APPROVAL PAGE

This study entitled "Characterization of HRP-based Amperometric Biosensor by using Screen Printed Electrodes" prepared by Mahmood Taha Noori AL-Sadoon under the supervision of Assist. Prof. Dr. EBRU SAATÇİ was accepted by the jury as M.Sc. Thesis in Biology.

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APPROVAL

That the acceptance of this thesis has been approved by the decision of the Institute's Board of Directors with the 15/01/2019..... date and ..2019/03-05..... numbered decision.

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Prof. Dr. Mehmet AKKURT

Director of the Institute

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Erciyes University, Graduate School of Natural and Applied Sciences M.Sc. Thesis, January 2019
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ABSTRACT

Immunosensors are examples of highly specific biosensors with excellent selective properties based on antigen-antibody interaction. In electrochemical immunosensors, the resulting current is the result of a function of either the concentration of direct electroactive species or the production or consumption rates in the biocatalytic material. When compared to other methods, high precision is a good alternative because of its advantages such as wide linear range of use, high stability, fast response and economic advantages. In immunosensors, classical ELISA methods are adapted on the biosensor.

The most commonly used enzyme in immunosensors is horseradish peroxidase (HRP). Different substrates of HRP are used in amperometric measurements. The HRP substrate, 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS), yields the oxidation and reduction peaks against the reciprocal voltammetry Ag/AgCl reference. Another HRP substrate, TMB (3,3)-5,5-Tetramethylbenzidine dihydrochloride) is used to determine the immobilized antigen concentration. The signal on the sensor generates an electroactive signal. Thus, it is used in the electrochemical determination by the amperometric biosensor.

Recently magnetic micro beads (MBs) are very attractive in the development and fabrication of sensors and biosensors for several applications. Screen-printing is one of the most promising approaches towards simple, rapid and inexpensive production of biosensors. The combination of these materials are very promising for the development of amperometric immunosensors.

In this study, conjugated enzyme (HRP) and its substrates (ABTS and TMB) were compared with amperometric measurements to understand their effects on

immunosensor development. For this reason, avidin-HRP binded biotin-IgG antibodies were immobilized on protein G-coated magnetic beads. From that point onward, ABTS or TMB was added to MB mixture and a constant potential was applied to the system. Measurrment are performed by using Dropsens 8x SPE carbon electrodes. For ABTS, the range of IgG antibody concentration was found between 5-360 ng / ml. For TMB, the IgG antibody linear standard curve was drawn and with the short biosensor detection time, the detection limit range was found between 0.1-20 ng / ml.

Keywords: Immunosensor, HRP, ABTS, TMB

HRP TEMELLİ AMPEROMETRİK BİYOSENSÖRÜN BASKILI ELEKTROTLAR İLE KARAKTERİZASYONU

Mahmood Taha Noori AL-Sadoon

Erciyes Üniversitesi, Fen Bilimleri Enstitüsü
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ÖZET

İmmünosensörler, antijen-antikor etkileşimi temelinde yüksek seçici özelliklere sahip oldukça spesifik biyosensörlerin örnekleridir. Elektrokimyasal immünosensörlerde ortaya çıkan akım, doğrudan elektroaktif türlerin konsantrasyonunun veya biyokatalitik malzemedeki üretim veya tüketim oranlarının bir fonksiyonunun sonucu değerlendirilmektedir. Diğer yöntemlerle karşılaştırıldığında, yüksek hassasiyet, geniş kullanım alanı, yüksek stabilite, hızlı tepki ve ekonomik avantajlar gibi avantajlarından dolayı iyi bir çalışma metodudur. İmmünosensörlerde klasik ELISA yöntemleri biyosensöre uyarlanır.

İmmünosensörlerde en sık kullanılan işaretleme enzimi, yaban turpu peroksidazı'dır (HRP). Amperometrik ölçümlerde farklı HRP substratları kullanılmaktadır. HRP substratı, 2,2'-Azinobis [3-etilbenzotiyazolin-6-sülfonik asit] -diamonyum tuzu (ABTS), enzim aktivitesi sırasında, Ag / AgCl referansına karşı oksidasyon ve indirgeme tepkimeleri vermektedir. İmmobilize edilmiş antijen konsantrasyonunu belirlemek için başka bir HRP substratı olan, TMB (3,3) -5,5-Tetrametilbenzidin dihidroklorür) de kullanılmaktadır. Sensör elektroaktif bir sinyal üretmektedir. Bu yüzden amperometrik biyosensörlerde elektrokimyasal ölçüm amaçlı kullanılmaktadır.

Son zamanlarda manyetik mikro boncuklar (MB'ler), çeşitli uygulamalar için sensörlerin ve biyosensörlerin geliştirilmesinde ve üretilmesinde kullanılmaya başlamışlardır. Baskılı elektrotlar ise basit, hızlı ve ucuz biyosensör üretimine yönelik en umut verici yaklaşımlardan biridir. Bu malzemelerin kombinasyonu amperometrik immünosensörlerin gelişimi için çok umut vericidir.

Bu çalışmada, konjuge enzim (HRP) substratlarının (ABTS ve TMB), immünosensör geliştirilmesi üzerindeki etkilerini anlayabilmek için amperometrik ölçümlerle HRP

aktivitesi karşılaştırıldı. Bu nedenle, avidin-HRP bağlanmış biyotin-IgG antikorları protein G-kaplı manyetik boncuklar üzerine immobilize edildi. Bundan sonar, MB karışımına ABTS veya TMB eklendi ve sisteme sabit bir potansiyel uygulandı. Ölçme, Dropsens 8x SPE karbon elektrotlar kullanılarak gerçekleştirildi. ABTS için, IgG antikoru limit aralığı 5-360 ng/ml arasında bulundu. TMB için, IgG antikoru lineer standart eğrisi çizildi ve kısa biyosensör algılama süresi ile limit aralığı 0,1-20 ng / ml arasında bulundu.

Anahtar Kelimeler: İmmünosensör, HRP, ABTS, TMB

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CHARACTERIZATION OF HRP-BASED AMPEROMETRIC BIOSENSOR BY USING SCREEN PRINTED ELECTRODES

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DISCUSSION AND CONCLUSION

LIST OF ABBREVIATIONS

ABTS : 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid.

ELISA : Enzyme-linked immunosorbent assay.

HRP : Horseradish peroxidase

MBs : Magnetic Beads.

SPEs : screen printed electrodes.

TMB : 3,3′,5,5′- Tetramethylbenzidine dihydrochloride.

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INTRODUCTION

Biosensors are newly developed special field which keeps on being an exceptional active region of actual research. They were characterized as gadgets that respond to chemical species in biological samples or biological components. They are now described as analytical devices composed of a biological recognition element, directly interfaced to a signal transducer, which together gives the concentration of targeted analyte or analytes. Therefore, the principle of a biosensor is the recognition of a target molecule or a specific event by a biological molecule on a transducer. A biosensor is depicted as "an optical biosensor for, an immuno-sensor for, an electrochemical geno-sensor for, and so on.

Electrochemical sensors are generally utilized because of their straightforwardness and scaling down to a gadget which is moderately simple. In addition, the investigation is quick, exceedingly delicate and requires minimal effort. Thus, electrochemical transducers are appropriate for the development of small and portable devices. They are used in many fields including food safety, green energy, bio-medical and environmental monitoring.

Electrochemical immunosensors are a decent contrasting option to traditional immunoassays. They utilize a biological componentsuch as, an Ab or antigen, immobilized on a transducer. They permit the perception and examination of the Ab-antigen association 'progressively', with or without the need for a name. Two primary configurations utilized for immunosensors are sandwich and competitive tests. It is significant that the strategy for immobilization type keeps up the solidness and movement of the biorecognition element.

Amperometry is one of the well-known electrochemical measurements, based on the measurement of the current intensity in the specific (defined) potential. In amperometric biosensors, the current intensity is a function of the concentration of the electroactive

species that is oxidized or reduced on the working electrode. By using a second electrode acting as a reference, the current intensity is used to determine the concentration of the species to be analyzed. Amperometric immunosensors depend on ELISA protocols.

ELISA (enzyme-linked immunosorbent assay) is a plate-based test procedure intended for recognizing and evaluating substances, such as, peptides, proteins, antibodies and hormones. In an ELISA, an antigen or an antibody is immobilized on a plate and after the complex formation between Ab and antigen, reading is done by enzyme labeled antibody. A detection enzyme or other tag can be connected specifically to the essential counter acting agent or presented through an optional immune response. It can likewise be connected to a protein, for example, streptavidin, which has an essential immune response with biotin. The most known detection enzymes are horseradish peroxidase (HRP) and alkaline phosphatase (AP). They have substrates (ABTS, TMB, OPD for HRP and p-NPP for AP) to get colored change for the detection of the analyte. The decision of substrate relies on the required test affectability and the instrumentation (spectrophotometer, fluorometer or luminometer).

Immunosensors are relatively related with ELISA tests. They include ag-ab interactions as well. This interaction is occurred on the transducer and a conjugated enzyme is used to obtain the biosensor signal, due to the electron transfer between oxidized substrate and reduced product.

In this study, the conjugated enzyme (HRP) substrates (ABTS and TMB) were compared with amperometric measurements to understand the effectiveness of both substrates on immunosensor production. For this purpose, avidin-HRP binded biotin-IgG antibodies were immobilized on protein G coated magnetic beads. After that, ABTS or TMB was added to the solution and a constant potential was applied to get the current change, due to the electron transfer between oxidized substrate and reduced product. All the measurements were done with Dropsens 8x electrodes.

CHAPTER 1

GENERAL INFORMATION

1.1. Biosensor

The definition of a biosensor is given by the National Research Council (US National Academy of Sciences) [1].

a) It has a living organism or product (eg, an enzyme or an antibody) and

b) An indicator that identifies the product obtained by a transducer which is defined as a detection device that carries out recognition in the presence of a particular substance with a signal in the environment.

On a fundamental level, biosensors are receptor-transducer based apparatus which could be utilized for translating the biophysical or biochemical property of the medium. The most charming character of biosensors is identification of specific natural molecules in the medium [2]. Advancement of biosensors is still continuing. On account of their high affectability, straightforward utilization, quick reaction, and similarity with miniaturization, they are broadly used in clinical detections, nourishment quality control, ecological checking and environmental monitoring [3]. The most advantages of biosensor are requiring minimal effort and composed of simple apparatuses which allow them to miniaturize.

A biosensor is composed of two parts: a bioreceptor and a transducer. Bioreceptor recognizes the analyte of interest, while the transducer changes the biochemical, optical, thermal response to a quantifiable signal. The most important component in the biosensor is the biocomponent. These biocomponents must have a selective character that will bind a single substrate and will not bind to different substrates. Binding should be sensitive enough but must be reversible. There are basically three groups of

bioreceptors; biocatalytic receptors (enzymes), biinfinite receptors (antibodies) and hybrid receptors (DNA) [4,5]. Structure and working principle of biosensors are given in (Figur1.1.) [6,7].

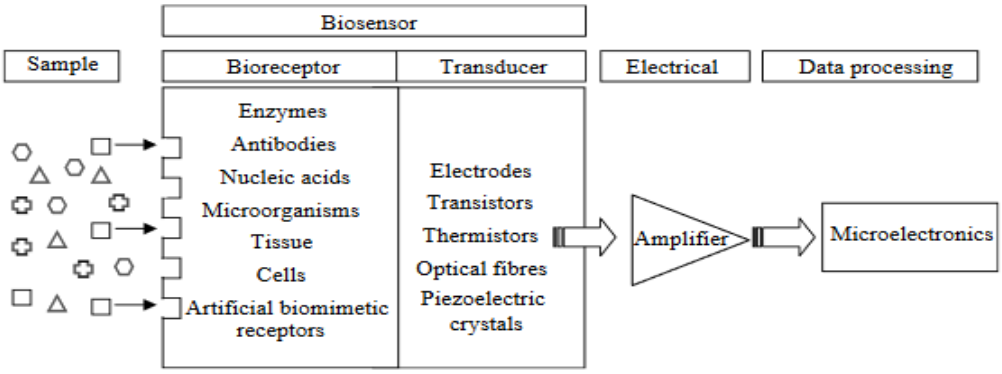

Figure 1.1. Structure and working principle of biosensors [8].

Depending on the type of signal, biosensors are divided into four groups:-as optical, calorimetric, piezoelectric and electrochemical . Optical biosensors are based on the principle of measuring light absorbed or emitted after a biochemical reaction. In such a biosensor, light waves travel to detectors with optical fibers. The calorimetric biosensors determine the presence of a temperature-dependent analyte due to the biochemical reaction of the analyte with a suitable biocomponent. Piezoelectric biosensors work according to the electric dipole principle, which is subjected to a natural anisotropic crystalline mechanical stress. The analyte adsorption increases the crystalline mass and changes the basic oscillation frequency of the crystal. All these biosensors suffer from certain disadvantages.For example, optical biosensors are very sensitive, but not fuzzy. Thermal biosensors cannot be used in systems with very small temperature changes [9].

Electrochemical biosensors are the most widely used biosensors today. In term of usage,these biosensors are simple then the others. When a bio-interaction process occurs on electrochemical biosensors, an electrical signal is generated due to the electrochemical species. The signal can be measured by an electrochemical detector. These biosensors can be used in fuzzy environments and have very high sensitivity. They are small in size and inexpensiv [9].

1.2. Biosensor Classification

Biosensors can be classified by either the nature of the bioreceptor component or the guideline of activity of the transducer[10].

1. Bioreceptor classification

According to bioreceptor part, they can be characterized into three main groups such as biocatalytic, bioaffinity and hybrid receptors [2, 3, 13].

a) Biocatalytic receptors: Biocatalytic recognition elements (e.g. enzyme-containing systems, cells, microorganisms such as bacteria, eukaryotic cells, yeasts, organelles and some tissue fragments are used to develop the system [9,11].

b) Bioaffinity receptors: They show determinative interactions that convert a selected ligand into a fixed complex form through a thermodynamic path [12]. Biological components such as antibodies, nucleic acids, lectins, dyes, cell membrane receptors and other specific binding agents are bioaffinity receptors. The detection and measurement of hormones, drugs, viruses, tumor antigens, bacterial antigens and many other protein-like analytes are achieved in minimal concentrations by using immunological methods [13,14].

c) Hybrid receptors: Usually, different macromolecules are immobilized on the biosensor surface. The use of nucleic acid in biosensor applications as hybrid receptors is indicated. Macro molecular interactions such as protein-DNA, aptamer-oligonucleotides, and DNA_RNA produce signals on the electrode surface. Uses of these sensors are applied to determine the DNA damage and the microorganisms by hybridizing the species with specific DNA sequence [11].

2. Transducer classification

According to transducer type, biosensors can be characterized into 5 main groups:

1) Chemical Biosensors

A chemical sensor is used to measure the amount of analyte in the sample directly. Such a sensor should ideally be able to operate continuously and reversibly without damaging

the sample. Chemical sensors contain a signal transducer element coated with bioreceptor. The chemical changes resulting from bioreceptor-analyte interaction are converted into electrical signals by the signal converting element. The electrochemical biosensor is a simple system in which electrodes carrying one or more biological catalysts. Electrochemical biosensors can be used in qualitative and quantitative analysis of various gases, ions and biological

substances and to monitor the original systems. With the development of the technology, various chemical and biological substances, which have electroactive properties, have been detected easier, faster and more sensitive. With the classical electrochemistry, only sensors that determine the anions and cations can be measured, while the addition of biomaterials to the system allows the determination of many other substances [1].

1.2.1. Electrochemical biosensors

Electrochemical biosensors are the first and most widely used biosensors. They were in the form of enzyme electrodes developed to detect clinical glucose in the early stages, especially by monitoring the oxygen. Next, enzyme-linked immunoelectrochemical studies have been designed by Heinemann et al. (1992) to increase the sensitivity of electrochemical biosensors and other studies are concentrated in this direction [15].

1.2.2. Amperometric Biosensors

The amperometric conversion is based on the measurement of the change of current generated by the electroactive product due to the electrochemical oxidation or reduction of the electroactive species. In the working method, a reference electrode is made by applying a specific potential to the platinum, gold, graphene or carbon working electrode. The resulting current is the result of a function of either the concentration of direct electroactive species or the rate of production or consumption in the biocatalytic material [15,16,17].

According to other methods, high sensitivity is a good alternative because of its advantages such as wide linear range of use, high stability, rapid response and economic availability [18]. The first biosensor developed for the monitoring of glucose is an amperometric .A general amperometric biosensor design is given in Figure1.2.

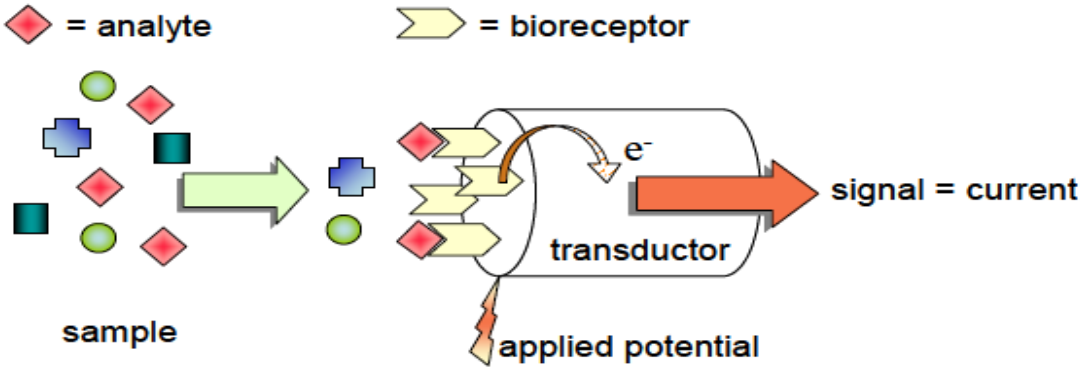

Figure 1.2. A general scheme of an amperometric biosensor [19].

Amperometric biosensors are very touchy and more suitable for large scale manufacturing than the potentiometric ones [20].The working electrode of the amperometric biosensor is normally either a respectable metal or a screen-printed layer covered by the biorecognition segment [21].The use of a potential between a reference and a working electrode empowers a current to be estimated at the point, when an electroactive analyte is oxidized or decreased, contingent upon the voltage at the counter electrode. The current (nA to μA) is identified with the rate of the electrochemical response that happens on the transducer [22,23].

1.2.3. Potentiometric Biosensors

Potentiometric biosensors are operated according to the ion selective electrode principle. The catalytic reaction of the biosensor can detect K^+, Ca^{+2}, Na^+, H^+ or NH_4 ions[24].

1.2.4. Conductometric Biosensors

These biosensors measure the conductivity change between a pair of metal electrodes. Various enzyme reactions such as many membrane receptors and urease can be monitored by ion conductometric devices using micro-electrodes. Therefore, the sensitivity in the measurement is proportional to the conductivity in the sample solution. The prepared biosensors allow the monitoring of electronic conductivity, such as the redox potential or pH around the polymer matrix [25].

1.2.5. Optical Biosensors

Optical biosensors are based on the evaluation of biocatalyst and target analyte with different optical principles and techniques such as absorbance, reflection, diffusion, luminescence and fluorescence. They are working by the lost area with the aim of detecting refractive index difference of light on the sensor [26,16].

1.2.6. Colorimetric Biosensors

In colorimetric biosensors, there is a system in which a chromogenic material is used to expose a color. Unlike optical recognition, a mechanical transducer is usually used with a naked eye. There are several trials that show fast and easily applicable environmental analyzes with the colorimetric based transducer mechanism for phenolic compounds and pesticides [27].

1.2.7. Piezoelectric Biosensors

Piezoelectric-based transducers are often administered in immunosensors, generated by the arrest of the antigen or antibodies to a crystal surface. The connection between these biological components and the analyte, the conversion of the monitored crystalline vibration to the signal is determined [15]. Normally, piezoceramics are utilized as actuators and polymer piezo films are utilized as detecting materials[28].

Numerous scientists have utilized piezoceramic sheet components as sensors in auxiliary frameworks [29] and furthermore in wellbeing observing applications [30]. The greater part of these applications depends on the relative extents of either the voltage or rate of progress by the sensor [29].

1.2.8. Thermal Biosensors

Biosensors with thermal transducers are based on the monitoring of energy difference in the form of heat in a chemical reaction, catalyzed by enzymes or microorganisms[13].

1.3. Immunosensors

Immunosensors are the example of highly specific biosensors with excellent selective properties, based on antigen-antibody interactions. Antigens and antibodies can be used as the biological component for the determination of each other. They are devices that comprise both a biomolecular acknowledgment system, such as, antibody,

its corresponding antigen, and a transducer. The interaction between the antigen and the antibody is produced a signal which is converted into an electrical signal by the transducer. Transducers recognize the adjustment in absorbance, fluorescence, refractive shift, mass electron or thermal changes. Immunosensors include electrochemical, optical, mass, and thermal structures as transducers [31,32].

Antibodies are used as bioreceptors since 1950s. They have been proven to have high sensitivity and selectivity. The most important feature of antibody-based biosensors is that the target can be used directly without the need for purification prior to detection [32].

Immunosensors are attractive in developing of quick and highly sensitive immunological response. Sensors are customary immunoassays. Generally, they are composed of enzyme linked immunosorbent assays (ELISAs) and electrochemical sensors [30].

Electrochemical ELISA, using enzyme-labeled antibodies, can be consideredas the most common type of amperometric immunosensor. The immobilization of the biological recognition element (antibody) on the surface of the transducer is the most common configuration to developthebiosensor. However, it is also possible to design different immobilization carriers such as magnetic beads that can be placed on the electrode surface by the application of magnetic fields. Thus, the antibodies can be fixed to the surface of the magnetic beads. Antibody-antigen formation is more effective and faster than direct electrode immobilization when using microparticles[33,34].

By developing immunosensors, various analytes (pathogens, poisons, ecological contaminants, cancer biomarkers and food additives can be recognized and measured [35].

1.4. Enzyme-Linked Immunosorbent Assay (ELISA)

The methods called EIA are distinguished as homogeneous and heterogeneous. In homogenous technique, the enzyme is conjugated to a hapten. The basis of this technique is based on the onset of enzyme activity in which the enzyme is conjugated with the antibody. However, technique has a disadvantage having to use low molecular weight substances and being expensive[36].

In heterogeneous EIA, bound and non-reactive reagents are physically separated from each other by washing. Enzyme linked immunosorbent assay (ELISA) is an example of a heterogeneous EIA. In ELISA, an enzyme, conjugated to an antibody (or antigen), reacts with its substrate to form a colored product[36]. It is a biochemical procedure, used in immunology to distinguish the nearness of an antigen or an antibody [35].

Although ELISA is not a complex technique, many variables should be checked. Solid phase immobilization, washing processes, selection and activity of the enzymes and substrates are done to develop an ELISA test. Plastic is mostly used as the solid phase to form microtiter plates. Apart from polystyrene microtitration wells, plastic beads, ferrous beads and nitrocellulose membrane can also be used as solid materials [37].

The enzymes (peroxidase, alkaline phosphatase, β-galactosidase) used in ELISA are well-defined enzymes in terms of their kinetics and their degree of conjugation. They are capable of converting substrates into colored products. These colored products can be taught in the standard spectrophotometer. If β-galactosidase is used, it should be read in the fluorimeter. Substrates are usually nitrophenyl phosphate for alkaline phosphatase and, ABTS and TMB for HRP [37].

ELISA might be keep running in a subjective or quantitative protocol. Subjective protocol generally outcomes with a positive or negative result. The cutoff amongst positive and negative is dictated by the investigator and might be factual. In quantitative ELISA, the optical absorbance or fluorescent units is measured and calculate to find the real concentration of interested analyte [38].

1.5. Types of ELISA

1.5.1.1. Direct ELISA

Direct ELISA was initially found by Perlman and Engvall (1971). The surface of the plate is directly covered with the antigen. An enzyme conjugated antibody is added in to wells. After washing step, the appropriate substrate of the enzyme is added and the color production is followed by a spectrophotometer (Figure 1.3). Absorbance signal is used for the calculation of the concentration of the analyte. This type is useful for the determination of high atomic weight antigens. It is thought to be the easiest type of

ELISA. Less progress is needed and faster than large-scale types of ELISA. Interaction of the secondary antibody is also eliminated [39].

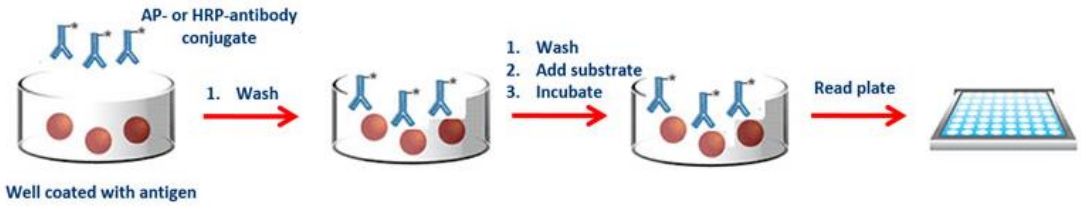

Figure 1.3. Schematic representation of direct ELISA protocol [39]

1.5.2. Indirect ELISA

In this technique, the primary antibody is incubated with the antigen-covered wells. Next, an enzyme conjugated secondary antibody is added to the wells. Then a substrate is added to deliver a colored signal enhancement (Figure 1.4). This strategy is generally used to analyze disease, caused by microscopic organisms, infection, or a parasite [39].

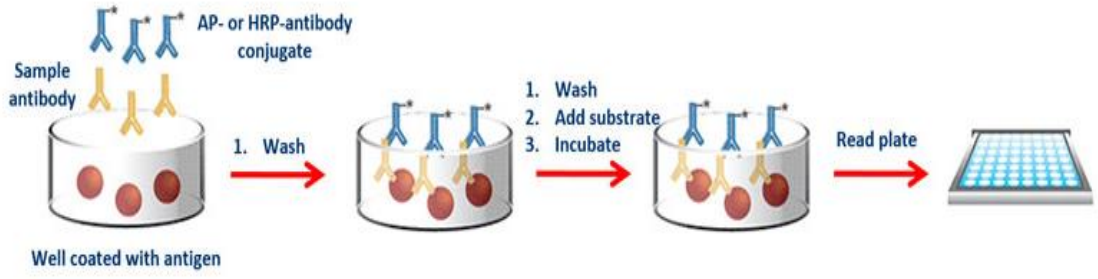

Figure 1.4. Schematic representation of indirect ELISA protocol [39]

1.5.3. Immunometric/Sandwich ELISA

Immunometric or sandwich ELISA utilizes two antibodies particular to the antigen to catch in a "sandwich" organized protocol. Firstly, microtiter plate is covered with a primary antibody. After that antigen is added to the wells and finally an enzyme-labelled secondary antibody is added. After the chromogenic substrate addition, the colored product is followed by spectrophotometer (Figure 1.5) [39].

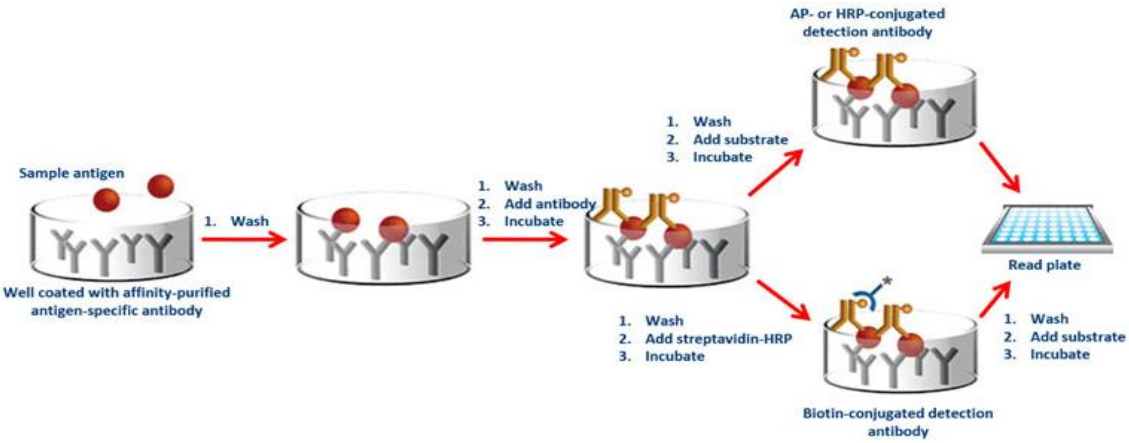

Figure 1.5. Schematic representation of sandwich ELISA protocol [39].

1.5.4. Competitive ELISA

In competitive enzyme immunoassay, sample antigen competes with an enzyme-labeled standard antigen to bind the primary antibody. Again, the chromogenic substrate addition, absorbance, coming from the colored product, is measured. Competitive ELISAs are developed for low molecular weight antigens under 10,000 Daltons, which cannot be detected by the other ELISA methods [39].

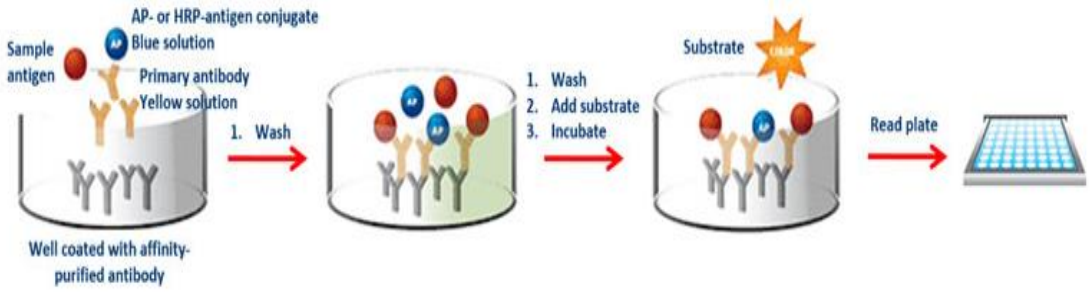

Figure 1.6. Schematic representation of a competitive ELISA protocol [39].

1.6. Antibody-binding proteins

Efficient immobilization of an antibody on the solid surface or a metal particle is an essential step in the preparation of immunosensors. A great variety of methods suitable for immobilization of antibodies has been described in the literature. Adsorption and chemical modification dependent on immobilization methods often suffer from random orientation and denaturation of immobilized antibodies, yielding poor reproducibility.

Due to these problems, recently antibody binding proteins are used for the conjugation of antibodies on the solid surfaces (electrodes) [40].

Orientation of surface immobilized capture antibodies plays a critical role in the performance of immunoassays. The sensitivity of immunosensors is dependent on capture direction of the antibodies, needs the antigen binding sites be directed toward the solution phase. Methods for improving antibody binding are including surface modification and design (with sections on surface treatments, three-dimensional substrates, self-assembled monolayers, and molecular imprinting, covalent attachment (including targeting amine, carboxyl, thiol and carbohydrates)), and (bio)affinity techniques (with sections on material conjugated with peptides, biotin-streptavidin interaction, DNA directed immobilization, Protein A and G, Fc binding peptides, aptamers , and metal affinity molecules) [41].

Protein A and G are well known Ab-binding proteins. These proteins bind particularly to Ab through antibodies non-antigenic (Fc) parts, which leave the antigen binding domain of the immobilized Ab accessible for antigens. Such Ab-binding proteins have been widely used to catch antibodies on biosensor surfaces (Figure 1.7).

Immunoassays have antibody-binding proteins for antibody immobilization regularly and exhibit higher sensing abilities compared to the other conventional methods. One of the major concerns associated with the method is the additional immobilization process for antibody-binding protein prior to antibody immobilization [42].

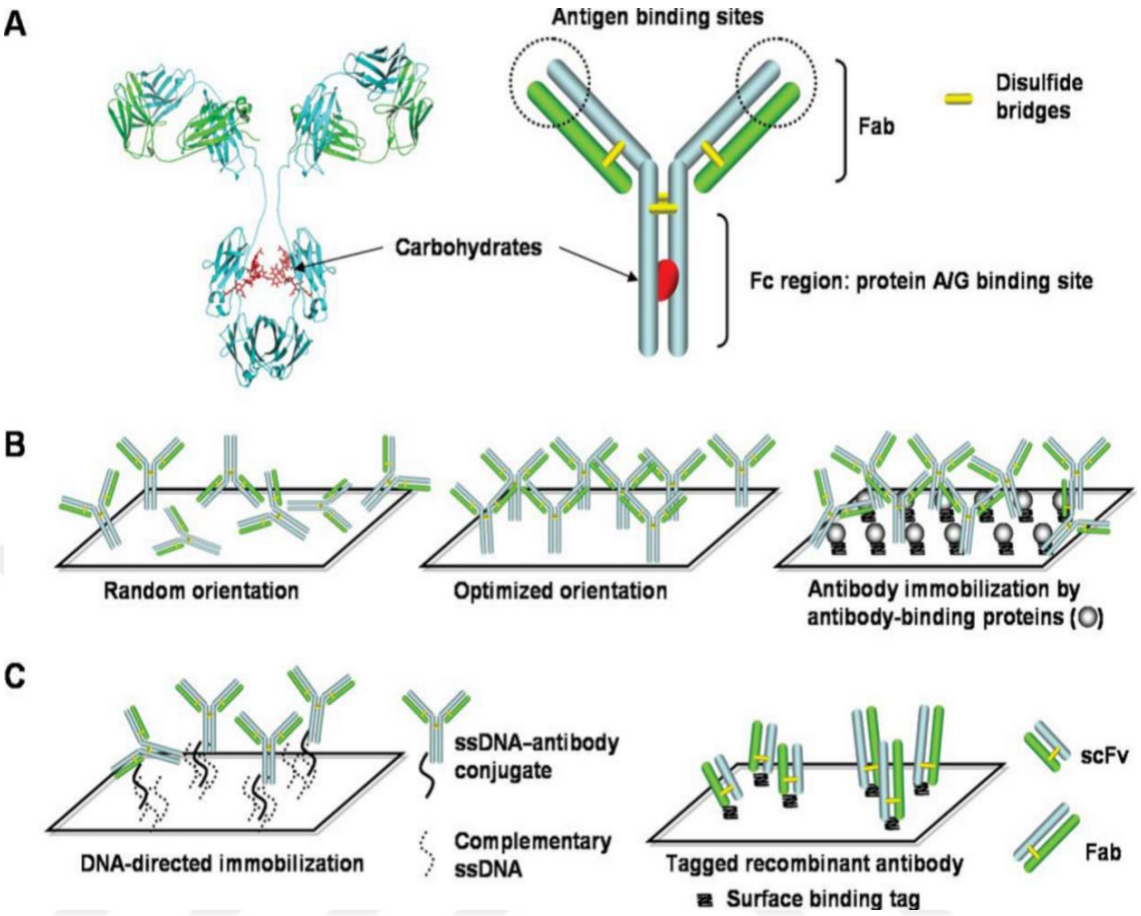

Figure 1.7. Schematic representation of different immobilization strategies done in immunosensors [42].

Protein G is a cell surface protein from which is an important member of IgG binding receptors on *Streptococcal* and *Staphylococcal* strains. It is well known that Protein G binds to Fc portion of IgG antibodies with its B1 domain. Although it has a high affinity to Fc domain, also it has affinity for Fab portion of antibodies. It is found that there is an interaction between secondary structural parts (outer β-strand) with protein G and Fab (Figure 1.8). These features of protein G allows purification of antibodies from various antibody containing mixtures [42,43].

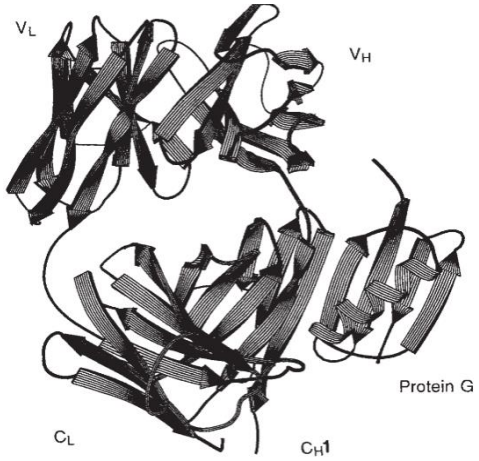

Figure 1.8. Overall structure of the Fab/protein G binding. Protein G interacts with the constant domain of the heavy chain (CH1) of antibody [43].

1.7. Enzymatic labels used in immunosensors

Enzymatic biosensors are not just used to recognize following the concentration of substances of interest, in addition they are used to examine enzymatic activities. They are well known in ELISA kits and amperometric immunosensors.

Peroxidases are used in catalyzing the oxygenation of a variety of organic and inorganic substrates by hydrogen peroxide or related compounds. The most commonly used peroxidase is horseradish peroxidase (HRP), catalyzes the oxidation of several compounds, e.g., ferrocyanide, phenolic compounds, o- and phenylene diamines, iodide, ascorbic acid, etc. In amperometric HRP-based immunosensor, the oxidization reaction product is turning in to electrochemically reduced molecule at an applied potential substantially lower than the direct oxidation of hydrogen peroxide [44,45].

1.8. Horseradish Peroxidase

Horseradish Peroxidase is a metalloenzyme that exists in the root of the horseradish plant. There are an expansive number of peroxidase isoenzymes with the most well-known being type C. Horseradish peroxidase utilizes hydrogen peroxide to oxidize both organic and inorganic compounds [46]. Peroxidase has two metallic groups: one iron in the heme group and two calcium atoms. The heme group contains a flat structure with an iron atom that is firmly anchored in the middle of the porphyrin ring consisting of four pyroles. It has two open binding positions, one above and none down on the plane

of the heme group. The group contains histidine, located under the heme group. This site is open to hydrogen peroxide to attach during oxidation reduction reactions. An oxygen atom will be connected to this vacant site during activation.

The site of the hexadecimal atoms of the iron atom is the active site of the enzyme. During the interaction of the enzyme, the hydrogen peroxide bonds to the iron atom, results in the formation of eight surfaces around the iron atom. Other small atoms can also be connected to the distant site making the same octahedral (composition) [46].

Figure 1.9shows the five oxidation states of HRP. There are three different intermediate horseradish peroxidase compounds that form during reactions. The reduction of compound I to compound II, back to the rest state, is carried out by the reduction of substrates(phenols or aromatic amines) [47].

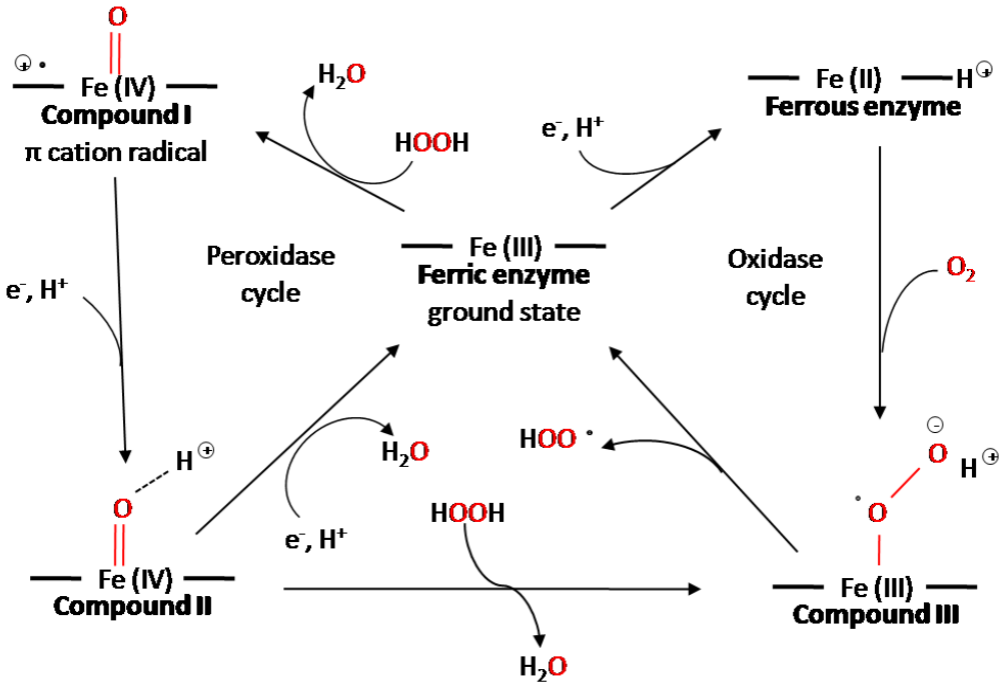

Figure 1.9. Reduction-oxidation cycle of HRP [47].

HRP is also the most widely used enzyme in the development of enzyme-based amperometric immunosensors. HRP-based immunosensors have easy availability in high purity and low cost. In order to reduce the reduction potential of H_2O_2, they are immobilized on electrodes to exhibit good specificity of enzyme reactions and high sensitivity of electrochemical transducer.

1.8.1. HRP Substrate Types

There are a few distinct sorts of HRP substrates. The kind of substrate utilized for identification relies upon the specific examine and method of location. Chromogenic HRP substrates become colored after reaction with HRP. Because of this, they are regularly used for ELISAs and other colorimetric measurements. Regularly utilized chromogenic HRP substrates are 3,3',5,5'- tetramethylbenzidine (TMB) and 2,2'-azino-di- [3-ethylbenzthiazoline-6-sulfonic acid] (ABTS). The choice of soluble chromogenic substrate depends on the requirements for assay sensitivity and the capabilities of the microplate reader [46].

1.8.2. ABTS 2,2-azino-bis(3-ethylbenzothiazoline-6sulfonate)

ABTS gives a well-known peroxidase-catalyzed reaction, one-electron oxidation of ABTS in to the corresponding radical–cation ($ABTS^{+}$). In the presence of excess of H_2O_2, peroxide are catalyzed oxidation of ABTS, lead not onlyto$ABTS^{+2}$, but also furtherrtoazodi cation$ABTS^{+2}$. $ABTS^{+2}$is unstable in aqueous solution; its decomposition products are pale-yellow or colorless. Both chemically and enzymatically generated $ABTS^{+2}$decomposes to the same five compounds in the mixture. These five products can be2+ (Figure 1.10) [48].

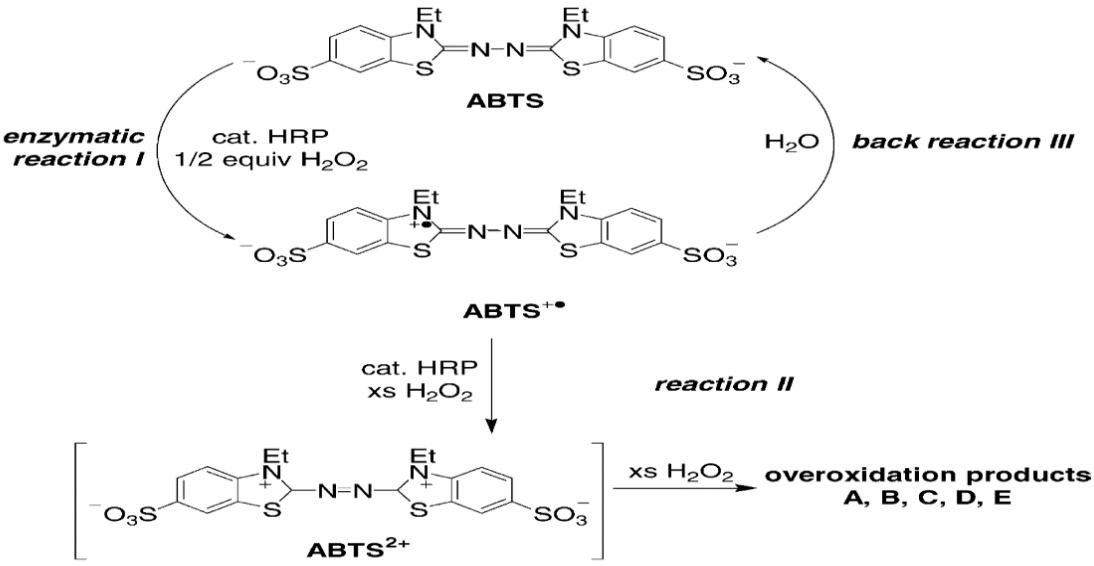

Figure 1.10. Oxidation of ABTS byhydrogenperoxide is catalyzed by horse radish peroxidase (HRP) encapsulated in sol–gel silicaglass [48].

ABTS is a good electron mediator that exhibits fast and effective electron transfer between the electrode and HRP. In the presence of H_2O_2, electrochemical oxidation of ABTS in solution takes place at an applied voltage (vs. Pt) to form $ABTS^+$ and $ABTS^{+2}$. Oxidation and reduction peaks produced from ABTS electrochemical reaction are observed at different voltages (vs. Ag/AgCl) and generally the reduction peak is taken by the signal of the biosensor [49].

1.8.3. TMB (3,3¢,5,5¢-Tetramethylbenzidine)

TMB was presented by Bos et al. in 1981 as a chromogenic substrate for HRP-based detection systems[50]. TMB is neither mutagenic nor carcinogenic[50]. and is more sensitive than traditional than HRP substrates like O-phenylenediamine (OPD) and 2,2¢-azino-bis-(3-ethylbenzthiazoline ethylbenzthiazoline-6-sulfonic acid) (ABTS)[51]. TMB exhibited initial problems related to its stability and poor solubility in aqueous buffer solutions; in any case, a few details have been proposed to enhance the solubility and to increase the sensitivity of this substrate [52,53]. Hence, these days TMB is the mostly used chromogenic substrate in HRP-based ELISA test kits [54].

Thus the mechanism of oxidation of TMB by HRP is a notable procedure which is presented by Josephy et al [55]. Oxidation of TMB by HRP with H_2O_2 first creates a blue-colored complex item, which turns into yellow product after the addition of sulfuric acid to the reaction media. This yellow product has been identified as a two-electron oxidation product (diimine), which is stable in acid solutions. It has a greatest absorbance peak at 450 nm and it is additionally electroactive, is allowing an electrochemical detection [53]. The oxidation of TMB in the presence of hydrogen peroxide is given by the following reaction scheme (Figure 1.11) [56].

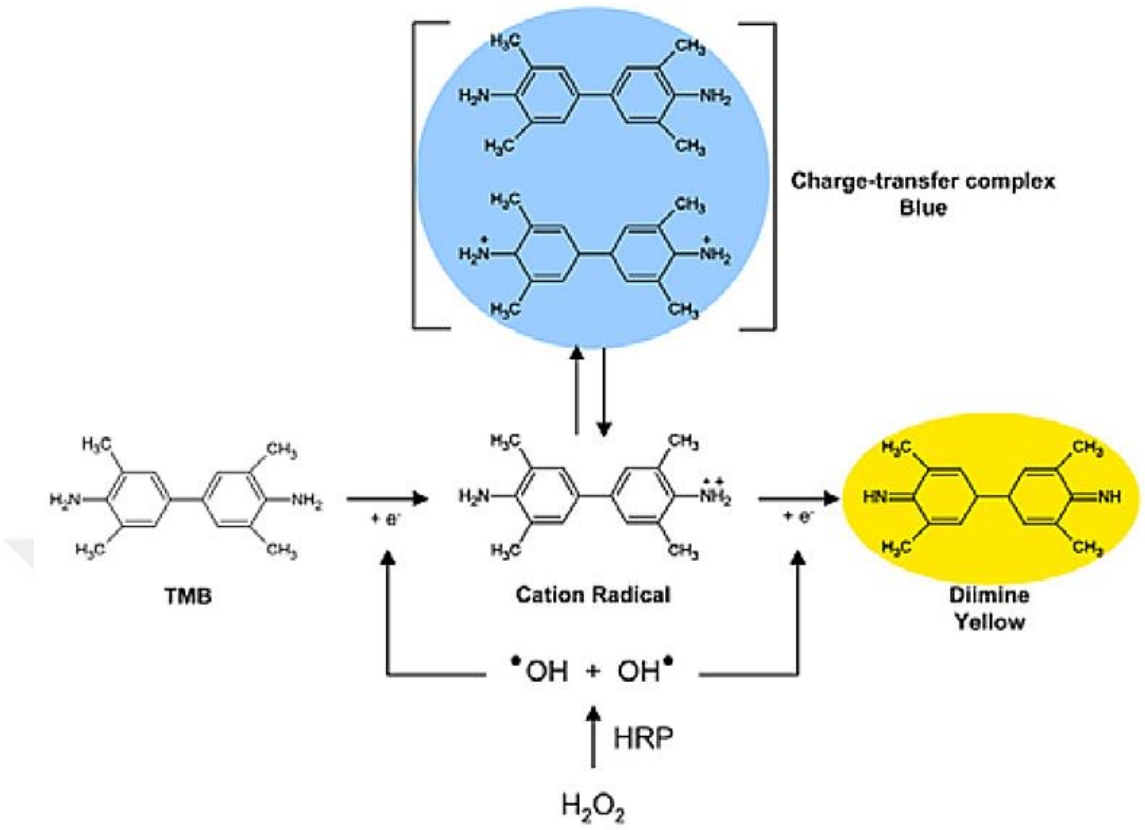

Figure 1.11. Chemical structure of TMB and its oxidation products[56].

TMB is also a good substrate for electrochemical detection of low levels of HRP. Cation radical produced from TMB is measured by amperometric detection in a constant applied voltage. Change in the current, related with the concentration of analyte of interest, is measured as biosensor signal [57].

1.9. Immobilization Methods

The process of connecting bioreceptors and transducers, are two important parts of biosensors, is called as immobilization. Immobilization is very powerful method for the re-usability and stabilization of the bioreceptor[58].Many effective techniques are used in the immobilization of biocomponents. The insolubility of the immobilized biocomponents allows them to be reused, controlled product formation and easy separation from the reaction. They also do not lose their efficiency during the reactions. Immobilization enables efficient use of biocomponents in food technology, biomedicine, biotechnology and analytical chemistry [59]. The most commonly used techniques arephysical adsorption, covalent bonding, cross-linking, capturing and microencapsulation (Figure 1.12).

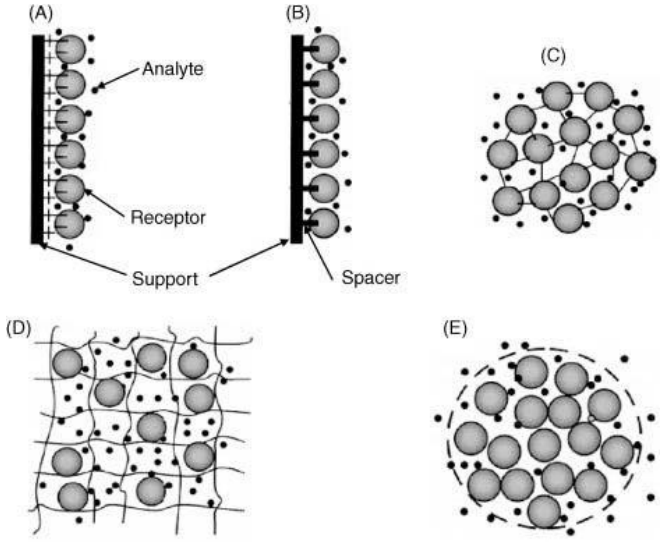

Figure 1.12. Methods used in immobilization of enzymes and other bioreceptors. A-Adsorption, B-Covalent Binding, C-Crosslinking, D-Capturing, E-Encapsulation [60].

1- Adsorption: In the physical adsorption, the biomaterial is attached to the surface by hydrogen bonds, van der Waals, ionic and hydrophobic forces. Cellulose, silica gel, glass and collagen are some known adsorbents. This method is the oldest and easiest method [58].

2. Covalent Coupling: The bioreceptor is binding to the transducer surface at the end of the chemical reaction. When enzymes are used as bioreceptors, binding takes place directly by the covalent binding to a transducer, a pre-film or a layer-coated transducer. This method is done by using the functional groups on the enzyme. Immobilization should be carried out under optimum conditions such as room temperature, neutral PH. Method increases the biosensor resistance to changes in temperature, pH, ion intensity and stability [58].

3- Capturing: Biomolecules are trapped within the polymeric gel matrix. Polyacrylamide, starch, alginate, pectate, polyvinyl alcohol, polyvinyl chloride, polyacrylamide, cellulose acetate and silica gel are generally used polymers. The disadvantage of the method is the possibility of leakage during the use of biological species and the result of the loss of the event [61].

4- Cross-linking: Bifunctional or multifunctional reagents such as glutaraldehyde, hexamethylene diisocyanate, difluorodynidobenzene, disuccinylsuberate are used for

cross-linking. Method has some disadvantages. Biological material and the occurred activity on the surface of the immobilized in multilayer enzyme formations is adversely affected [61].

5- (Micro) Encapsulation: An immobilized membrane is used for immobilization of the biomaterial on the transducer. The close relationship between the biomaterial and the transducer makes the method more applicable and reliable among the other methods [62].

1.10. Magnetic Beads

Different materials are used for the preparation of electrode surfaces in biosensor applications[63]. Glass and other oxide surfaces are commonly used materials due to their favorable properties[64]. In addition, gold[65]. microporous gold, graphite[66]. glassy carbon[67]. and indium tin oxide are commonly used materials [51].

Nano and microparticles are submicron fragments made from organic or inorganic materials. Their size is usually between 1 micron and 1 nm[68]. Magnetic microparticles are used in the development of biosensors and electrochemical sensors due to their unique electronic and chemical properties. Different types of particles have been used for various electrochemical detection systems such as enzyme sensors, immunosensors and DNA sensors[69]. Generally, the metal particles have excellent conductivity and catalytic properties, making them suitable for increasing the electron transfer between the redox centers of the enzyme and the electrode surfaces. The basic functions of the particles can be summarized as follows:

1- To facilitate immobilization of biomolecules (mostly oxide metallic particles)

2- Catalysis of electrochemical reactions

3- Improving electron transfer over increased surface area

4- Labeling of biomolecules

Magnetic beads are well-fixed materials, showing super magnetism [70,71]. Because of these properties, they are used in many different applications [72,73,74].

Recently their use in biotechnology has become very popular. Some advantages of immobilization of biocomponent on magnetic magnetic beads are:

1- They provide highly specificity surface area for more biocomponent binding.

2- They provide less contamination and low weight transfer resistance [75].

The most advantage of magnetic beads in biological applications are immobilization of biomolecules in an easy way [76]. For this purpose, the surface layer is designed to contain suitable bonding groups. Antibodies, nucleic acids and affinity reagents (eg streptavidin) can be conjugated with modified magnetic beads, covered with active groups such as carboxyl, amine, thiol or carbonyl. Abiotic receptors such as macrocyclic ligands, cryptaners or calixarenes may also bind in the same manner. The receptor interacts with the target analyte attached to the magnetic particles. Unbound analytes are then easily washed off and removed. The presence of the target in the detection layer is shown in the separated magnetic field induced by the particles. For example, in the immunosensor-based sandwich immunoassay model, the antibody binds to the magnetic bead bearing the different ligands and the antigen is bound to the specific region of the antibody (Figure 1.13) [77].

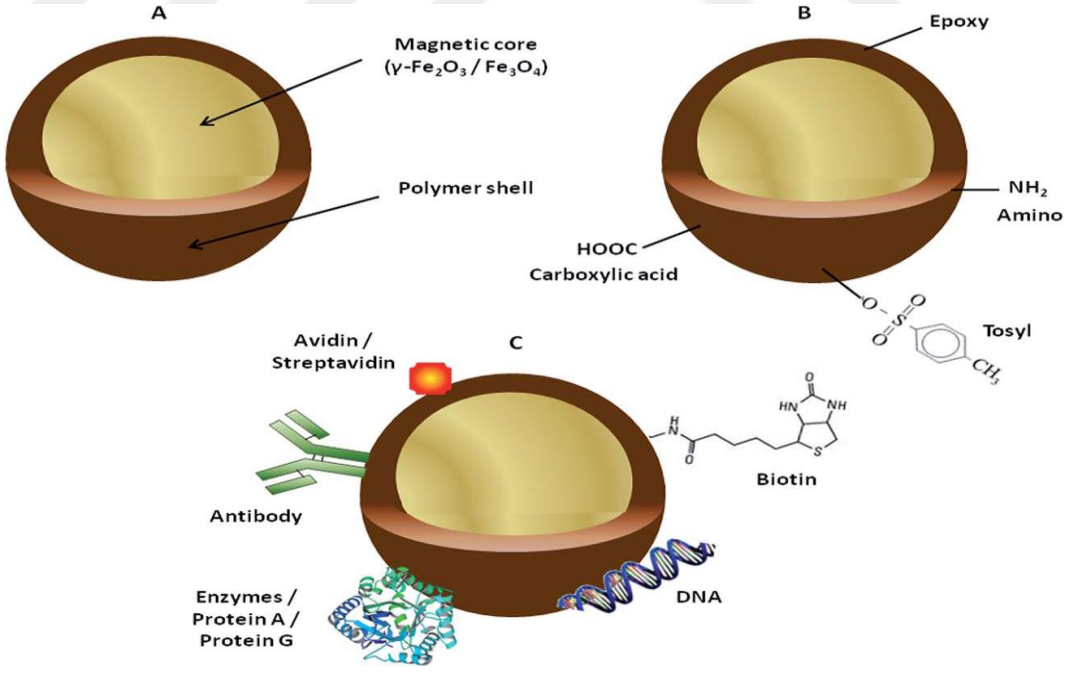

Figure 1.13. Schematicre presentation of magnetic particles (A), activated with functional groups (B) and conjugated to biological molecules (C) [77].

1.11. Screen-printed electrodes (SPEs)

Today scaling down is an obvious pattern of Analytical Chemistry. In the field of electroanalysis, the interfacial nature of measurements supports the fabrication of miniaturized analytical devices. In this circumstance, thin and thick-film technology is an awesome utility. In thick film technology, one of the well-known methods is screen-printing (SP), generally done for the large-scale manufacturing of expendable electrochemical sensors [78].

First investigations on thick-layer graphite disposable electrodes (called GDEs) are starting from 1995. Over the past twenty years, the screen printing of electrodes (SPE) and sensors is a major branch of electrochemical research, with hundreds of documents being published which refer to the technique [79].SPEs are portrayed for various applications, for example, amperometric detection, depends on peroxidase-based redox reaction coupling [79].

SPEs are electrodes produced by printing different conductive inks on various types of ceramic surfaces or insulating plastics [80]. In a small strip, the working electrode, the reference electrode and the auxiliary electrode are arranged around a common center (Figure 1.14) [81].The auxiliary electrode operates as a cathode when the working electrode is operated as an anode with an opposite response. The auxiliary electrode is usually produced from electrochemically inert materials such as gold, platinum or carbon [82].The reference electrode has two common types, Ag/AgCl and Hg_2Cl_2 electrode [82]. Materials such as gold, silver or carbon are also used in working electrode for the construction of SPEs in analysis and detection of various analytes [83]. Graphite materials are preferred in the construction of reference and working electrodes due to low cost and simple technological production [84].

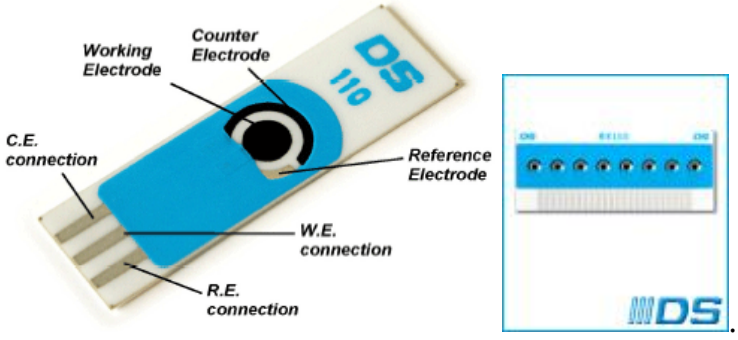

Figure 1.14. 1x and 8x Dropsens SPEs configurations [85].

The working electrode is where the reaction takes place and the immobilized bioactive layer is present. The density of the electroactive species oxidized or reduced on the working electrode is defined as the current density. Current amountis used for determination the concentration of the respective species in amperometric detection methods [86,87].

SPEs are widely used in the development of amperometric biosensors. Because they are cheap and can be produced on large scales and structure of SPEs can potentially reduce the risk of contamination in single-use sensors and prevent loss of sensitivity [82].In our study, we used 8x multiple Dropsens SPEs, which have typical electrochemical cell configuration. They are suitable for developing micro-volumes and design specific sensors. Also, they are very useful in the simultaneous detection of multiple analyzes.

1.12. Performance Factors of Biosensors

Stability: The length of shelf life of the biosensor is expressed by its stability. The longevity of a biosensor indicates the feasibility of working with the same material. This offers advantages in terms of cost and labor. In the case of enzyme sensors, the situation should be evaluated in terms of both sensor and biocomponent stability. When looking at the stability of the enzyme sensor, parameters such as the source of the enzyme, the level of purity and the immobilization method are important. Working in environment with lower temperatures increases the shelf life of the biosensor if it meets the requirements for working conditions [88,62].

Selectivity: Selectivity is the measure of detection without being affected by other species in the sample matrix. Interferences with pH, biocatalyst and sensor are effective in the selectivity of a biosensor. Amperometric sensors are more selective than other electrochemical sensors [89].

Repeatability: A series of test results over a period of time indicate the compatibility of the results [90]. Repeatability is also a measure of ordinary or unknown errors. Variance, variance coefficient and standard deviation are used in the calculation [91].

Working Range: The linear study range is obtained by transferring the analyte concentration of the biosensor to the difference in the electrode-response and it is linear to a certain analyte concentration. This range covers the range from the detection limit

to the linearity limit[90]. In potentiometric enzyme sensors, the calibration graph is obtained by plotting between the logarithm of the product concentration and it's potential. In amperometric enzyme sensors, linear graphs are obtained between the substrate or product concentration and the current. All graphics are also included in the Michaelis-Menten equation[62].

Response Time: It is the time that elapsed from the time of contact with the medium to be analyzed [88]. The response time differs in biosensors from a few seconds to minutes. While the values up to 5 minutes are accepted, 10 minutes is seen quite long [62].

Other Factors: Cost is one of the important factors affecting the performance of a biosensor. The costing the construction of a biosensor and the expenses of the study are expressed as total cost. The fact that analysis costs are less than other analytical methods is an important factor in the availability of the biosensor. However, having features such as not requiring pre-preparation can cause cost to be in the background when the sampling is dangerous. The transport of the biosensor to be used is particularly important in some special areas such as the environment [89].

In this thesis, HRP substrates (ABTS and TMB) were compared with an immune system. The reduction and oxidation potentials in the presence of H_2O_2 were studied by an amperometric biosensor. In order to understand the best substrate, avidin-HRP conjugated IgG immobilized protein G-magnetic beads were put on a carbon-based SPE and a constant potential was applied to the system. According to HRP concentration, change in the current was detected as the signal of the biosensor.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Chemicals used in study are fromMerck, Aldrich, Fluka, Sigma, Abcam, Abnova, eBioscience, MiliporeAmicon, Dynabeadsand Thermo in analyticalgrades.

2.1.2. Materials and Devices

Thematerialsanddevicesaregiven in Table 2.1, Figure 2.1 andFigure 2.2.

Table 2.1. Materialsanddevicesused in experiments.

Dropsens–DRP-Cast8X
Dropsens-DRP-Cast96x
Dropsens-8x potentiostat
Magnetic holder
Vortex mixer-Labnet İnternational, Inc.
pH meter-Mettler Toledo
Hotplate& Stirrer-HSD 180 MTOPS
Millipore distilled water supplier-Q-POD-Lod No: F4CA33864
Working buffer powder (Phosphate buffer, NaCl-0.138M; KCl-0.0027M; pH 7.4
Protein G coated magnetic beads (invitrogen)
Biotin -labeled affinity purified ntibody to Rabbit IgG (H+L)
Super Aqua Blue ELISA Substrate (ABTS)
3,3',5,5'-Tetramethylbenzidine (TMB)

Figure 2.1. Magnetic holder with magnetic beads.

Figure 2.2. Printed ECELISA system: Potantiostat, 8x electrode, connections and computer (with Dropview software)

2.2. Methods

Amperometry is one of the electrochemical methods in electrochemical biosensors which can be combined with ELISA techniques. The detection is based on the measurement of a steady-statecurrent in an electrolytic cell which is due to the oxidation or reduction of the analytspecies. In the presence of an applied potential, the current change from the oxidation or reduction of reactants or products is measured on a working electrode and the signal will depend on the concentration of analyte in solution. When we combine the method with a well-known immunological method (ELISA), we are getting a very sensitive and specific interaction for the determination of analyte of interest (antigen) by using specific antibodies as a biorecognition element. Generally, in a common ELISA test, enzymatic labels are used as the final step to get an absorbance from a colored product. In electrochemical immunosensors, the most common enzymatic label is horseradish peroxidase (HRP) which is also used in common ELISA

tests. Our experimental design depends on a similar HRP dependent electrochemical immunosensor, explained at below. General scheme of the experimental protocol was given in figure 2.3.

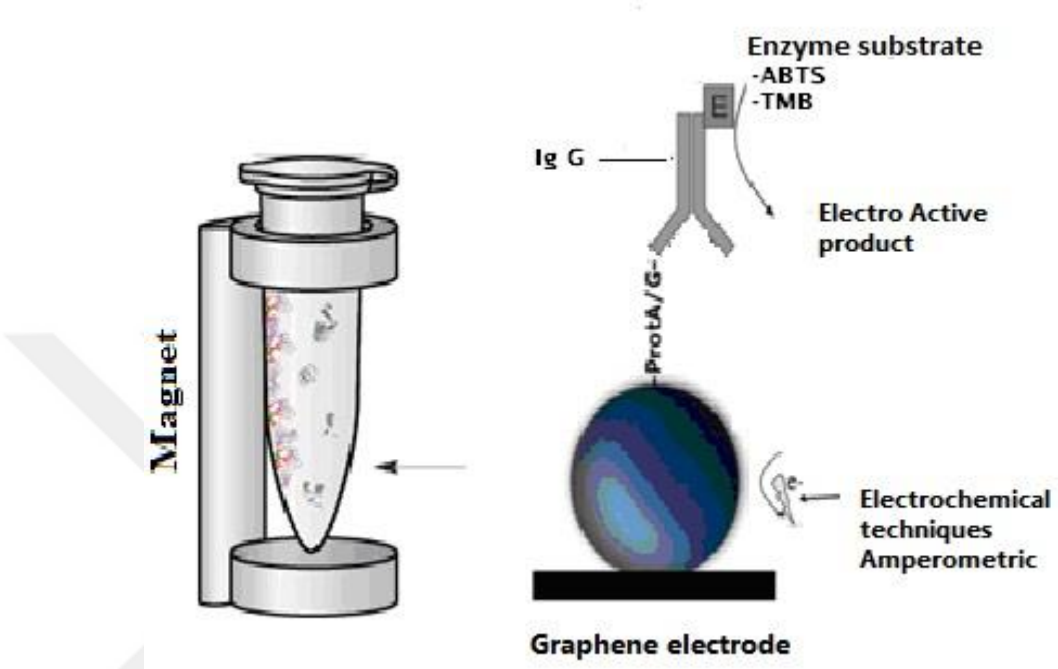

Figure 2.3. Protocol design of developed HRP-dependent immunosensor.

In this study, protein G-coated magnetic beads were used for conjugation of biotin-coated Ig G. Avidin-labeled HRP was conjugated with Ig G. After all these steps, HRP immobilized MBs and HRP substrates, ABTS and TMB were put on the carbon-based SPE. A constant potential was applied to the system via a multichannel potentiostat. Current change was followed by Dropview software. Working buffer was 0.01 M PBS at pH 7.4.

Working procedure in general:

Step1. Magnetic bead preparation

From 30 mg/ml MB stock, 1,5 mg/ml protein G-coated MBs were prepared according to MB protocol. During this step, MBs were washed with working buffer (0.01M phosphate buffered saline, NaCl – 0.138M, KCl – 0.0027M, Tween 0.05%, pH 7.4 at 25^{o}C) for three times on magnetic holder.

Step 2. Biotinylated IgG with avidin-HRP binding on protein G-MBs

Biotinylated IgG and Avidin-HRP were added into MBs and mixture was washed with working buffer on magnetic holder after 45 min incubation. Conjugated MBs were collected by using magnetic holder. Assuming that, IgG was bound on protein G from its Fab portion.

Step 3. Substrate addition

After the conjugation step, HRP bonded magnetic beads were put on 8x Dropsens SPEs and optimized electroactive substrate of HRP (ABTS or TMB) was added in MBs mixture on 8x electrodes by completing the total volume to 20 µl.

Step 4. Working potential application

A constant voltage was applied to system by using multichannel potentiostat and difference in the current (nA) was screened on a computer with Drop Views software. Current change was recorded and accepted as the biosensor signal.

2.2.1. ABTS Optimization Methods

2.2.1.1. Applied Potential Optimization

In order to find the system application voltage, amperometric measurements were done at 150mV application potential. This voltage was chosenaccording to Ferak (2017) results [92]. The amount of MB and biotin-coated Ig G was used as 0.48 mg/ml and 0.24 µg/ml, respectively. 3.8 mmol / l ABTS + 1.46 mmol / l H2O2, and 1/50 diluted HRP were in the reaction mixture. Readings were performed after 20 min incubation.

2.2.1.2. Biosensor Response Time Optimization

Activity readings were done at 10, 20_ and 30_min. Antibody, MB and avidin-HRP concentrations were 0.24 µg/ml, 0.48 mg/ml and 1/50 dilution, respectively. The substrate concentration was 3.8 mmol / l ABTS + 1.46 mmol / l H_2O_2. The application potential was 150 mV.

2.2.1.3. Optimization of Magnetic Bead Concentration

The effect of protein G-coated MB concentration was performed by adding different amounts of MBs into mixture. For this purpose, MBs at 0,06, 0,12, 0,24, 0,48 and 0,64mg / ml concentrations were put into mixture. The amount of biotin-coated Ig G was 0.24 μg/ml. 3.8 mmol / l ABTS + 1.46 mmol / l H_2O_2 and 1/50 diluted avidin-HRP were in the reaction mixture. Application potential was 150 mV and readings were performed after 20 min incubation.

2.2.1.4. Optimization of biotinylated-IgG

Different IgG concentrations (0,005, 0,02, 0,06, 0,12, 0,24, 0,36, 0,48 μg/ml) were added into the MB mixture. MBs and avidin-HRP concentrations in the measurements was 0,48 mg/ml and 1/50 dilution, respectively. 3.8 mmol / l ABTS + 1.46 mmol / l H_2O_2were put on the reading mixture. Application potential was 150 mV and readings were performed after 20 min incubation.

2.2.1.5. Optimization of Avidin-HRP concentration

Different avidin-HRP concentrations (1/50, 1/100, 1/200, 1/300 dilutions) were added into the MB mixture. MBs and biotin-coated Ig G concentrations in the measurements were 0,48 mg/ml and 0.24 μg/ml, respectively. 3.8 mmol / l ABTS + 1.46 mmol / l H_2O_2 were put into the reaction mixture. Application potential was 150 mV and readings were performed after 20 min incubation.

2.2.1.6. Optimization of ABTS and H_2O_2 Concentrations

For this purpose, different concentrations of ABTS and H_2O_2(0.76 and 0.292 mmol/l (1/5 dilution), 1.9 and 0.73 mmol/L (1/2 dilution) and 3.8 and 1.46 mmol/L (undiluted)) were prepared as HRP substrates. MBs and biotin-coated Ig G concentrations in the measurements were 0,48 mg/ml and 0.24 μg/ml, respectively. Application potential was 150 mV and readings were performed after 20 min incubation.

2.2.2. TMB Optimization Methods

2.2.2.1. Applied Potential Optimization

In order to find the system application voltage, amperometric measurements were done at 50, 100, 150 and 250mV application potential. The amount of MB and biotin-Ig G was used as 0.48mg/ml and 24 ng/ml, respectively. 0.4 mg/ml TMB and 0,02% $H_2O_2$and 1/50 diluted HRP were in the reaction mixture. Readings were performed after 5min incubation.

2.2.1.2. Biosensor Response Time Optimization

Activity readings were done at 5, 10, 15_and 20_min. Antibody, MB and avidin-HRP concentrations were 24 ng/ml, 0.48 mg/ml and 1/50 dilution, respectively. Substrate concentration was 0.4 mg/ml TMB and 0,02% H_2O_2. The application potential was 150 mV.

2.2.1.3. Optimization of TMB and H_2O_2 Concentrations

For this purpose, different concentrations of TMB and H_2O_2(0.08 mg/ml TMB and 0,004% H_2O_2 (1/5 dilution), 0.2 mg/ml TMB and 0,01% H_2O_2 (1/2 dilution) and 0.4 mg/ml TMB and 0,02% H_2O_2 (undiluted)) were prepared as HRP substrates. MBs and biotin-IgG concentrations in the measurements were 0,48 mg/ml and 24 ng/ml, respectively. Application potential was 150 mV and readings were performed after 5 min incubation.

2.2.1.4. Optimization of Avidin-HRP concentration

Different avidin-HRP concentrations (1/25, 1/50, 1/100, 1/200 dilutions) were added into the MB mixture. MBs and biotin-coated Ig G concentrations in the measurements were 0,48 mg/ml and 24 ng/ml, respectively. Substrate concentration was 0.4 mg/ml TMB and 0,02%H_2O_2. Application potential was 150 mV and readings were performed after 5 min incubation.

2.2.1.5. Optimization of Biotinylated-IgG

Different IgG concentrations (0,05, 0,01, 0,5, 1, 5, 10, 20, 40, 60 ng/ml) were added into the MB mixture. MBs and avidin-HRP concentrations in the measurements was 0,48 mg/ml and 1/50 dilution, respectively. Substrate concentration was 0.4 mg/ml TMB and 0,02% H_2O_2. Application potential was 150 mV and readings were performed after 5 min incubation.

CHAPTER 3

RESULTS

In this study, HRP substrates ABTS and TMB dependent activity studies were done by Printed-ECELISA device. In this context, electrochemical measurement was made by enzymatic signals, HRP. The protocol was based on the protein G-IgG and avidin-biotin interactions and ECELISA method was developed over MB and SPEs.

The signals received from ABTS and TMB were compared for the substrate sensitivity and detection limits of the newly established system. The conditions were first determined by direct immune measurements. By using 8x electrode screen printed carbon electrochemical electrodes (8xSPCE) as measurement system, results were evaluated and enzyme-based immunosensor design was completed.

The biosensor design was made for the optimization of last step of immunosensors (enzymatic labels) by conjugation of HRP to MBs.

3.1. HRP Substrate ABTS Results

3.1.1. Applied Potential Optimization

Applied potential was chosen from the latest work of the study group optimization results. Ferak (2017). found that two different oxidation and one reduction peak potential according to the cyclic voltammetry voltamogram. The potentials were found as 0.340 (I), 0.738 (II) and -0.294 (III) mV, respectively. The system potential was chooses as 150 mV[92].

3.1.2. Biosensor Response Time Optimization

Activity readings were done at 10, 20_ and 30_min. Antibody, MB and avidin-HRP concentrations were 0.24 μg/ml, 0.48 mg/ml and 1/50 dilution, respectively. The

substrate concentration was 3.8 mmol / l ABTS + 1.46 mmol / l H_2O_2. The application potential was 150 mV.

Under optimized conditions above, the optimum reaction time with the substrate of the HRP, ABTS, was determined. Even though the change in the current during time was increased, 20 min reading time of the enzyme with ABTS were considered as the optimum reading time. The reason for this is that the nA change amounts obtained at 20 min were sufficient enough for the experimental results (Figure3.1). Figure also gives the HRP dilution effect on activity reaction.

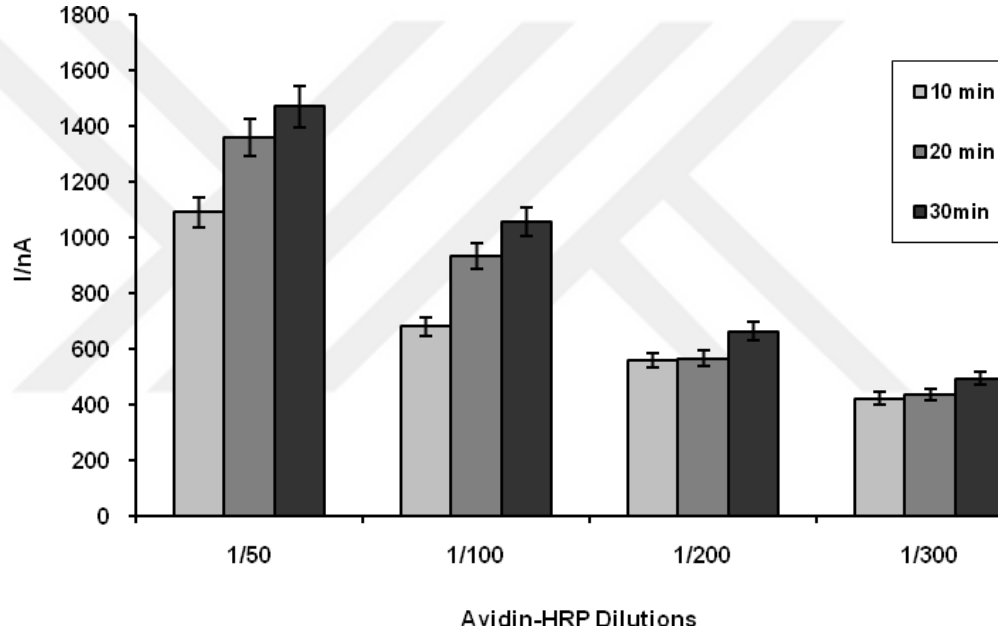

Figure 3.1. HRP activity reading time with ABTS as substrate.

3.1.3. Optimization results of Magnetic Bead Concentration

The effect of protein G-coated MB concentration was performed by adding different amounts of MBs into mixture. For this purpose, MBs at 0,06, 0,12, 0,24, 0,48 and 0,64 mg / ml concentrations were put into mixture. The amount of biotin-coated Ig G was 0.24 µg/ml. 3.8 mmol / l ABTS + 1.46 mmol / l H_2O_2 and 1/50 diluted avidin-HRP were in the reaction mixture. Application potential was 150 mV and readings were performed after 20 min incubation (Figure 3.2).

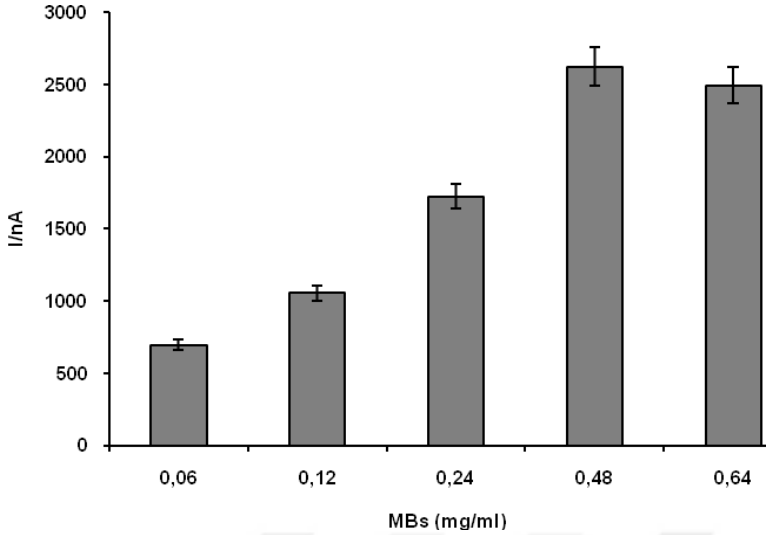

Figure 3.2. Optimization graph of protein G coated magnetic beads concentration.

According to results, 0,48 mg/ml MBs concentration was chosen as optimum amount for the biosensor measurements.

3.1.4. Optimization of Avidin-HRP concentration

Different avidin-HRP concentrations (1/50, 1/100, 1/200, 1/300 dilutions) were added into the MB mixture. MBs and biotin-coated Ig G concentrations in the measurements were 0,48 mg/ml and 0.24 µg/ml, respectively. 3.8 mmol / l ABTS + 1.46 mmol / l H_2O_2 were put into the reaction mixture. Application potential was 150 mV and readings were performed after 20 min incubation. (Figure 3.3)

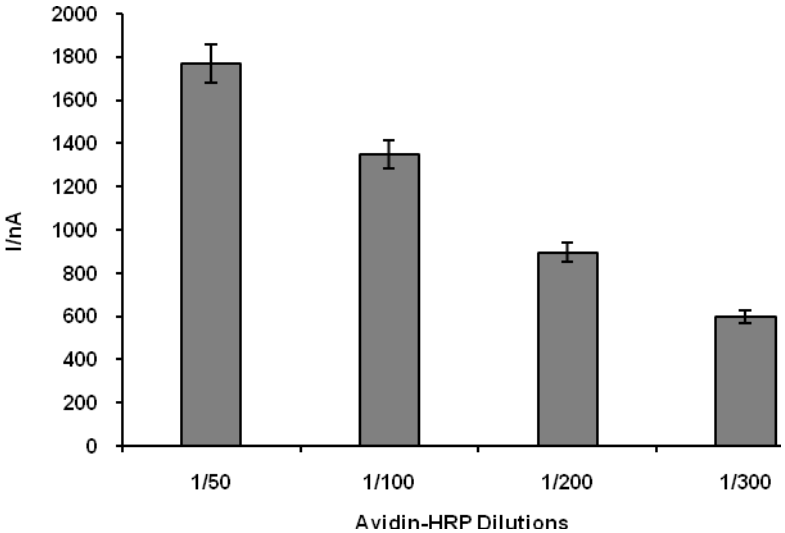

Figure 3.3. Avidin-HRP concentration optimization graph

As it is seen on the graph, optimum avidin-HRP dilution was choosen as 1/50 due to the maximum current change.

3.1.5. Optimization of ABTS and H_2O_2 Concentrations

For this purpose, different concentrations of ABTS and H_2O_2 (0.76 and 0.292 mmol/l (1/5 dilution), 1.9 and 0.73 mmol/L (1/2 dilution) and 3.8 and 1.46 mmol/L (undiluted)) were prepared as HRP substrates. MBs and biotin-coated Ig G concentrations in the measurements were 0,48 mg/ml and 0.24 µg/ml, respectively. Application potential was 150 mV and readings were performed after 20 min incubation.

Maximum current change was obtained from undiluted ABTS and H_2O_2 concentration (3.8 and 1.46 mmol/L). This was accepted as optimum amount. (Figure 3.4)

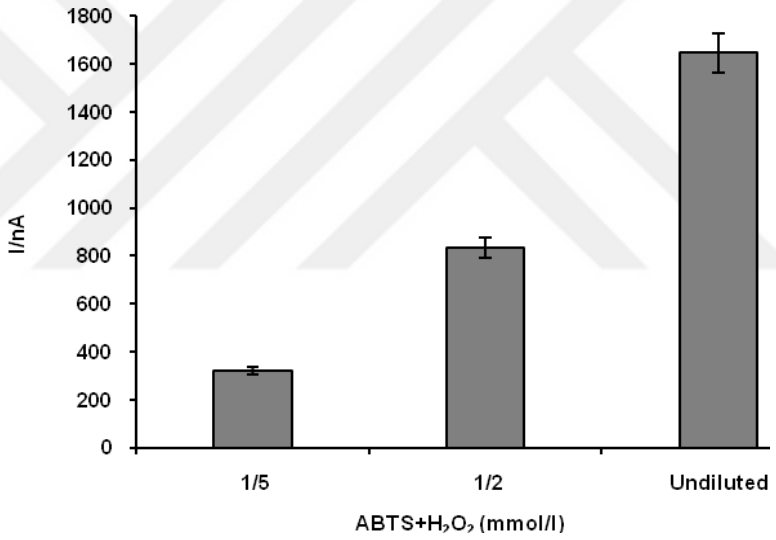

Figure 3.4. ABTS and H_2O_2 concentration differences in current vs concentration graph

3.1.6. Optimization results of biotinylated-IgG conjugation

Different IgG concentrations (0,005, 0,02, 0,06, 0,12, 0,24, 0,36, 0,48 µg/ml) were added into the MB mixture. MBs and avidin-HRP concentrations in the measurements was 0,48 mg/ml and 1/50 dilution, respectively. 3.8 mmol / l ABTS + 1.46 mmol / l H_2O_2 were put on the reading mixture. Application potential was 150 mV and readings were performed after 20 min incubation (Figure 3.5)

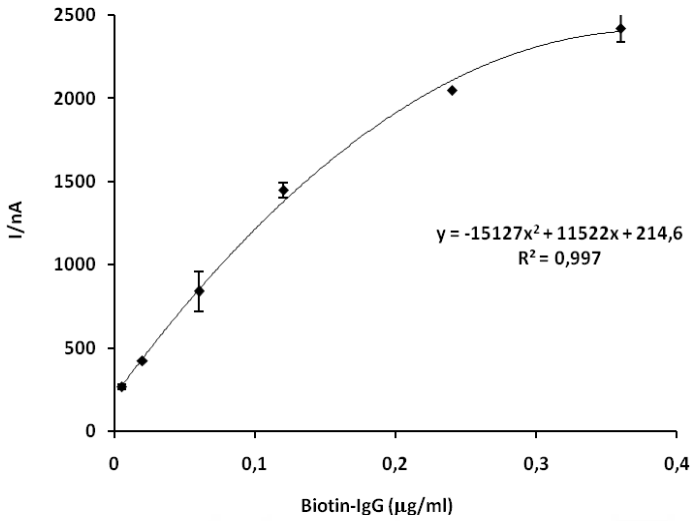

Figure 3.5. Biotin-Ig G standart graph with ABTS as the substrate

As shown in the graph, the standard graph shows a hyperbolic curve. While the concentrations of antibodies were reduced during the study, this was unchanged. Therefore, the point at which the slope changes (0.24 µg / ml) was considered to be the optimum antibody amount.

3.1.7. Amperometric chromatogram of IgG concentrations with ABTS

Amperometric voltamograms of the biotin-IgG standard graph were given in Figure 3.6

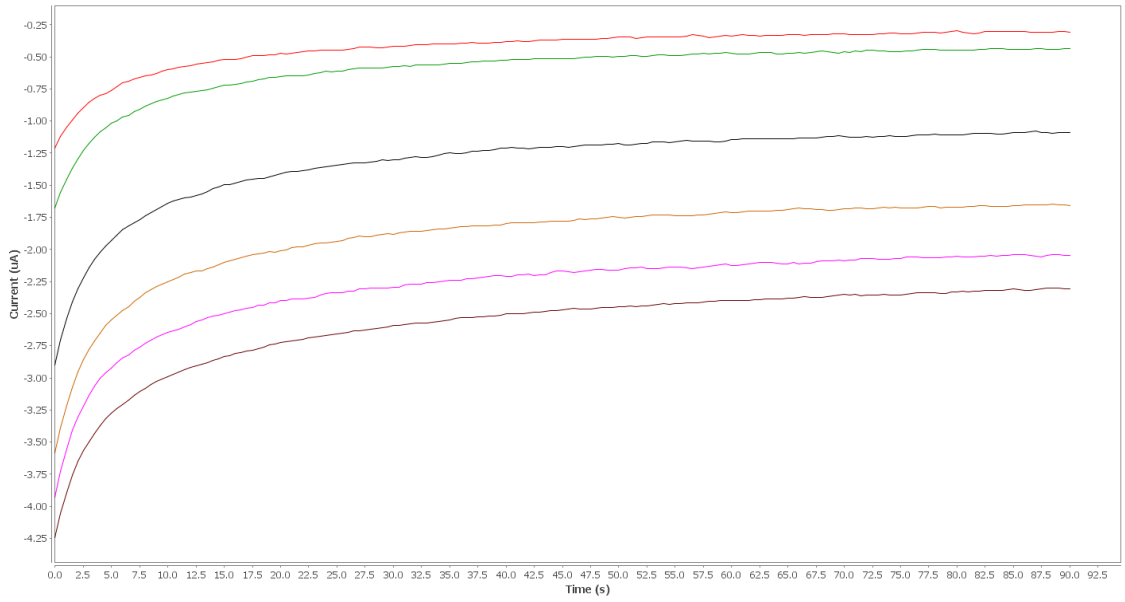

Figure 3.6. Amperometric voltamogram (Current vs time)of different concentrations of biotin-IgG (0,005-0,48 µg/ml) with ABTS

3.2. TMB Optimization Methods

3.2.1. Applied Potential Optimization

In order to find the system application voltage, amperometric measurements were done at 50, 100, 150 and 250 mV application potential. The amount of MB and biotin-Ig G was used as 0.48 mg/ml and 24 ng/ml, respectively. 0.4 mg/ml TMB and 0,02% H_2O_2 and 1/50 diluted HRP were in the reaction mixture. Readings were performed after 5 min incubation. In order to the maximum current change, applied voltage was chosen as 150 mV (Figure 3.7).

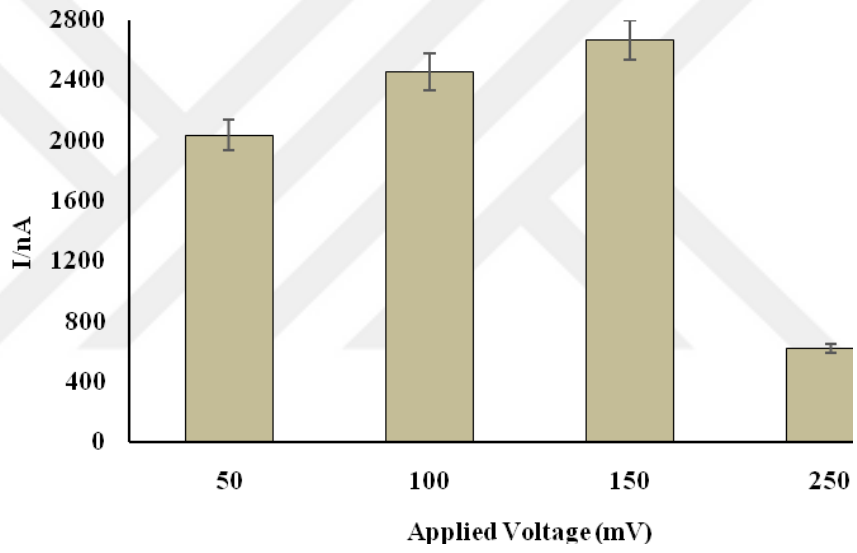

Figure 3.7. Hydrodynamic voltametry of TMB as substrate

3.2.2. Biosensor Response Time Optimization

Activity readings were done at 5, 10, 15- and 20-min. Antibody, MB and avidin-HRP concentrations were 24 ng/ml, 0.48 mg/ml and 1/50 dilution, respectively. Substrate concentration was 0.4 mg/ml TMB and 0,02% H_2O_2. The application potential was 150 mV.

Under the optimized conditions above, the optimum enzymatic reaction time with HRP substrate TMB was determined. Because of the change in the current during time was decreased,5 min reading time of the enzyme with TMB were considered as the optimum reading time (Figure 3.8).

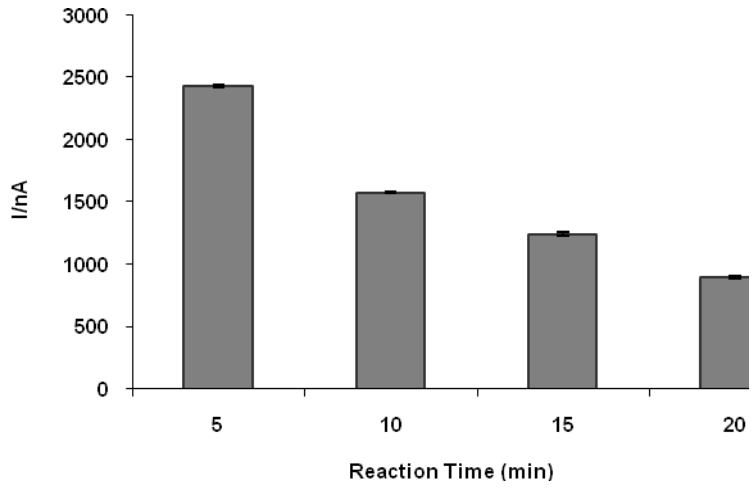

Figure 3.8. HRP activity reading time with TMB as substrate.

3.2.3. Optimization of TMB and H_2O_2 Concentrations

For this purpose, different concentrations of TMB and H_2O_2 (0.08 mg/ml TMB and 0,004% H_2O_2 (1/5 dilution), 0.2 mg/ml TMB and 0,01% H_2O_2 (1/2 dilution) and 0.4 mg/ml TMB and 0,02% H_2O_2 (undiluted) were prepared as HRP substrates. MBs and biotin-coated Ig G concentrations in the measurements were 0,48 mg/ml and 24 ng/ml, respectively. Application potential was 150 mV and readings were performed after 5 min incubation.

Maximum current change was obtained from undiluted TMB and H_2O_2 concentration (0.4 mg/ml TMB and 0,02% H_2O_2). This was accepted as optimum amount. (Figure 3.9)

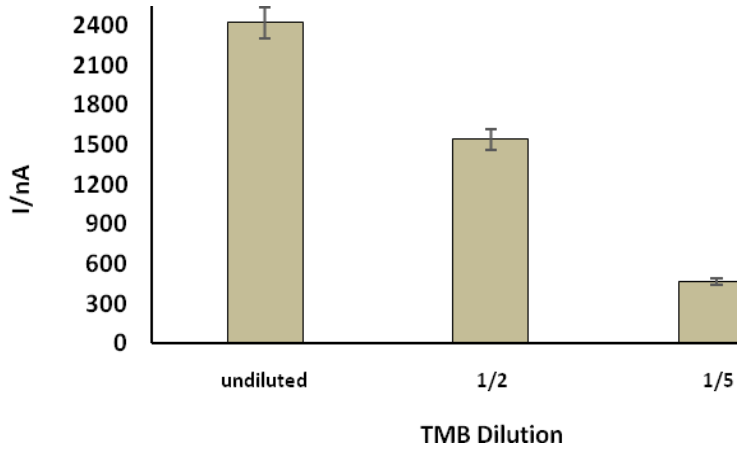

Figure 3.9. TMB concentration differences in current vs concentration graph

3.2.4. Optimization of Avidin-HRP concentration

Different avidin-HRP concentrations (1/25, 1/50, 1/100, 1/200 dilutions) were added into the MB mixture. MBs and biotin-coated Ig G concentrations in the measurements were 0,48 mg/ml and 24 ng/ml, respectively. Substrate concentration was 0.4 mg/ml TMB and 0,02% H_2O_2. Application potential was 150 mV and readings were performed after 5 min incubation.

As it is seen on the graph, optimum avidin-HRP dilution was choosen as 1/50 due to the maximum current change. (Figure 3.10)

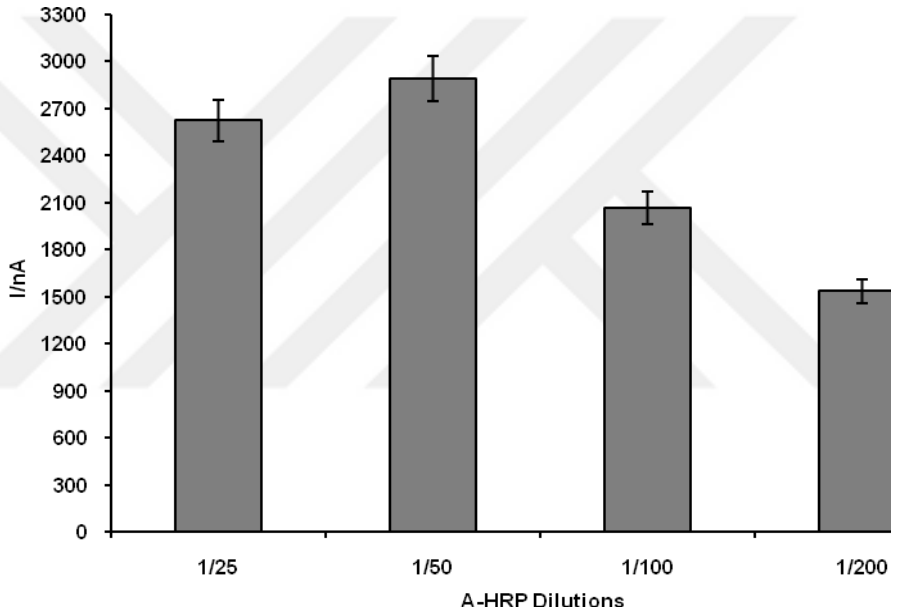

Figure 3.10 Avidin-HRP concentration optimization graph

3.2.5. Optimization of biotinylated-IgG detection limits

Different IgG concentrations (0,05, 0,01, 0,5, 1, 5, 10, 20, 40, 60 ng/ml) were added into the MB mixture. MBs and avidin-HRP concentrations in the measurements was 0,48 mg/ml and 1/50 dilution, respectively. Substrate concentration was 0.4 mg/ml TMB and 0,02% H_2O_2. Application potential was 150 mV and readings were performed after 5 min incubation.

As shown in Figure 3.11the standard graph shows a hyperbolic curve. Because of this,the concentrations of antibodies were reduced during the study and the linear standard graph was obtained from this reduction. 24 ng/mlwas considered to be the optimum antibody amount.

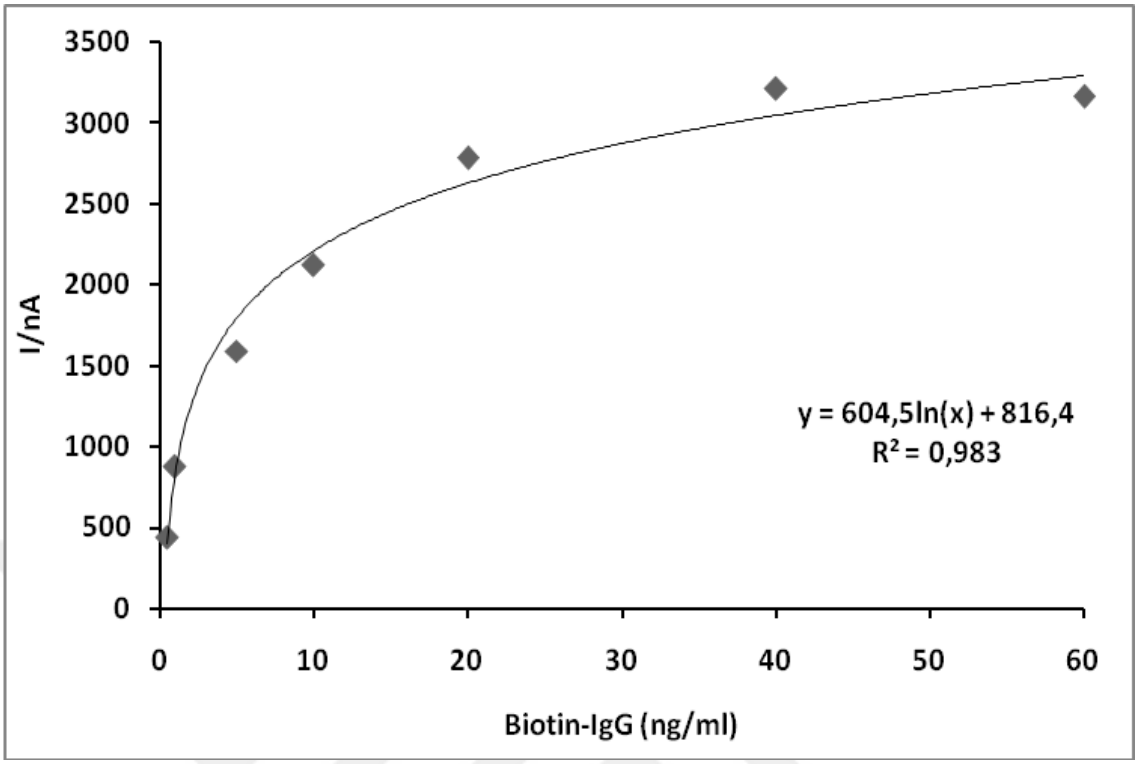

Figure 3.11. The effect of Biotin-Ig G concenration (0,1-60ng/ml) on the activity with ABTS as the substrate.

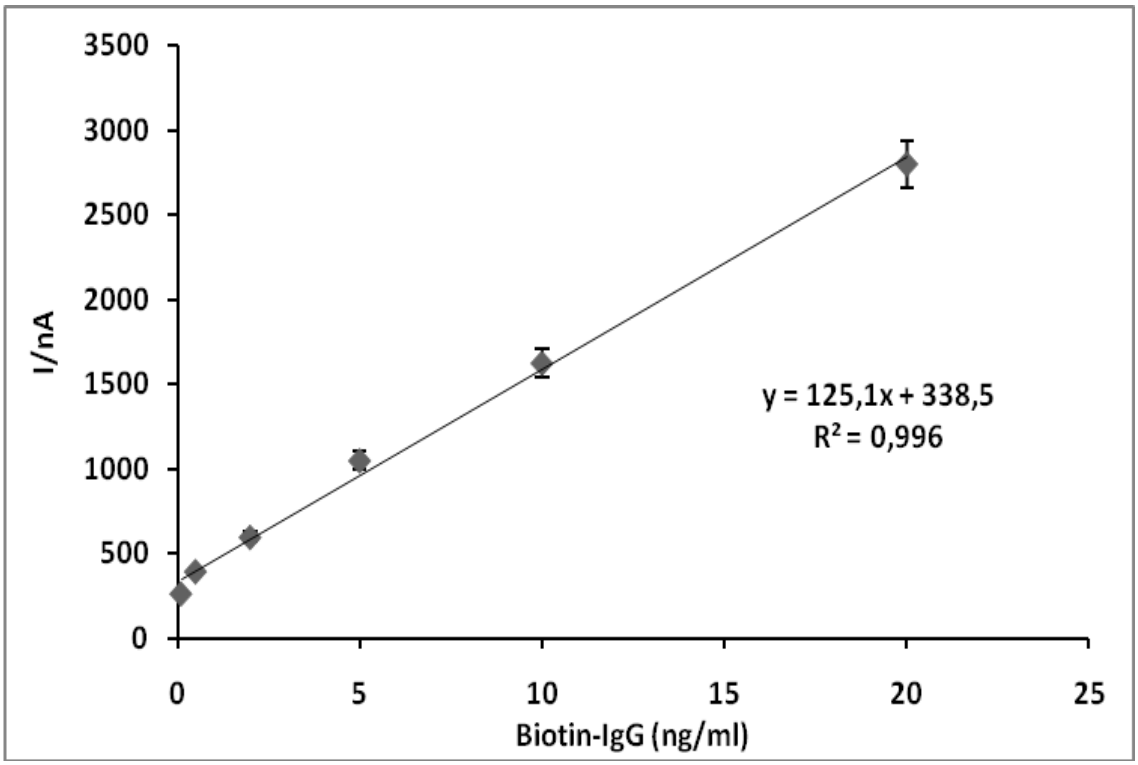

Figure 3.12. Biotin-Ig G standart graph with TMB as the substrate (0,1-20 ng/ml).

3.3. Amperometric chromatogram of IgG concentrations with TMB

Amperometric voltamograms of the biotin-IgG standard graph with TMBwere given in Figure 3.13

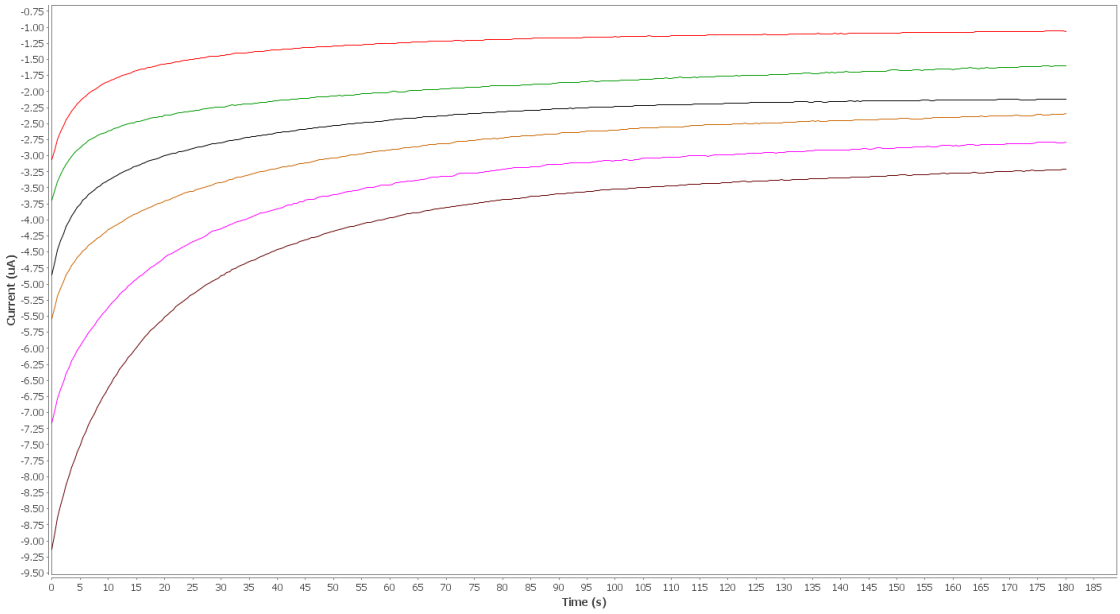

Figure 3. 13. Amperometric chromatogram (Current vs time) of different concentrations of biotin-IgG (0,1-20 ng/ml) with TMB

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1. Discussion

Immunosensors are based on interactions between an antibody and antigen on a transducer surface. Because of the strong binding between these molecules, they are highly selective and sensitive [89]. Immunosensors generally finished with enzyme activity detections, based on selective conjugated enzyme. Enzyme activity detection step is the last for the immunosensor design. Even antigen or antibody can be labeled with the selected enzyme. Enzyme activity measurement is performed after this step [54].

Immunosensors are also very useful tools for the development of electrochemical biosensors. Therefore, they can easily convert in to a portable sensor. Due to this property, they are generally disposable and can carry out in situ or automated detection [89]. Amperometric electrochemical detection has an excellent sensitivity and wide linear range. Detection method has attracted attention in recent years, with the development of electrochemical enzyme-based immunoassays [54].

Horseradish peroxidase (HRP) is the most commonly used enzyme label for amperometric immunoassays, has been studied for decades [54, 90]. The HRP labels are conventionally used in optical method-based measurements. Chromogenic substrates such as 3,3′5,5, -tetramethylbenzidine (TMB), 2′azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) generate a colored reaction product. These substrates achieve the detection limit ranging from 10−19 to 10−13 mol of HRP [90]. In conventional ELISA methods, researchers show that as a chromogenic substrate, TMB is more sensitive than ABTS. But it has some disadvantages, such as its

stability and poor solubility in aqueous buffer solutions. However, TMB is the most used in HRP-based commercially available ELISA test kits [91].

Oxidation of TMB by HRP/H_2O_2 produces a blue-colored complex product. This is also electroactive thus allowing an electrochemical detection [93]. Common HRP substrates developed for electrochemical HRP-labeled bioassays are TMB and ABTS [90].

The crucial step in development of an electrochemical immunosensor is the selection of the most efficient immobilization of biomolecules onto the electrode surface. Nowadays, classical immobilization methods are left and instead of these, newly developed micro and nanomaterial technologies have been opened up due to the disadvantages of classical immobilization procedures. Classical ones affect the properties of the sensing surface itself or cause the electrode surface through non-specific adsorptions. Micro technology is an advance technology to immobilize biocomponent on the surface of the working electrode in biomolecular detection. When it is combined with screen-printed electrode (SPEs) technology, it becomes more efficient, rapid, selective and sensitive for the determination of the target analyte [94]. SPEs can have carbon, gold or silver working electrodes. Carbon is the more reasonable electrode for studying with micro beads (MBs). Electrode has attractive properties such as a lower residual current leading to higher signal/noise responses and correspondingly lower detection limit. This combination allows the easy modification of the surface and reduces the sample mixture (only few microliters). Also, MBs are allowed us to make immobilization of biocomponent on a separate support[95,96]. In these cases, the electrode only acts as a measuring device. The only problem is that the recognition element is not in direct contact with the surface electrode. To avoid this, small equipment with magnetic parts is used for stabilization of the MBs on the surface of the SPE electrodes. By using this strategy, biosensor will get greater sensitivity and concentrating the MBs on the electrode surface for the final measurement[97,98,99].

A large number of biomolecules can be immobilized on the surface of each MB, because of their small size and spherical geometry. This improves the immunosensor type, method, sensitivity, short response time and use smaller volumes of solution on the working electrode surface[100,101,102]. Evaluation of electroanalytical

performances, the effectiveness of the combination is very useful for the modification and their potential use for electrochemical detection [103].

In this study, an amperometric biosensor based on immune measurement and based on HRP enzyme measurement was developed. With this sensor, different substrates of HRP, ABTS and TMB, were compared in terms of their characteristics. For this purpose, biosensor was developed with magnetic beads and SPE combination.

Many studies based on the selection of HRP substrate were selected according to the results obtained by colorimetric studies. If we compare our results with other amperometric biosensor studies, we see that our study is supported by other study results. At the same time, this study is the first study with the designed system.There is no study comparing two substrates with such a system at the same time.

In our thesis, HRP substrates ABTS and TMB independent activity studies were done by Printed-ECELISA device. In this context, electrochemical measurements were made for HRP activity determinations. The protocol was based on the protein G- biotinylated IgG and avidin-HRP interactions. ECELISA method was developed over MBs and SPEs. The signals received from ABTS and TMB were compared for the substrate sensitivity and detection limits of the newly established system. By using 8xSPCE as the measurement system, results were evaluated and enzyme-based immunosensor design was completed. The biosensor design was made for the optimization of the last step of immunosensors (enzymatic labels) with comparison of HRP substrates.

Our work potentials for ABTS and TMB are consistent with other studies[101,103,104,105]. Researchers applied working potential between 0,1 and 0,2 mV with similar systems on a carbon electrode. Our applied potential was 0,15 mV which stays between these potentials. Considering our results, we used to apply the same potential for ABTS and TMB. We showed that both of the substrate activities can be measured with the same potential.

Biosensor response was found as 5 min and 20 min for TMB and ABTS, respectively. These are also similar to other studies. Zor et al (2012). were found the response time as 25 min for ABTS[106]. and Bolado (2005). and Biscay (2014). were found the response time as 1 min for TMB[101,105].

When it comes to comparing our own results, TMB is more sensitive substrate than ABTS for HRP activity determinations. The reaction time was also significant, where this value was 5 min for TMB and 20 min for ABTS. Also, sensitivity was increased when we compare the IgG detection limit results. We found the detection limit as ng for TMB (0,1-20 ng/ml) and µg for ABTS (0,005-0,48 µg/ml). As a result, it is shown that using TMB for HRP substrate is more convenient than ABTS usage in future studies.

4.2. Conclusion

In conclusion, HRP enzymatic conjugate, plays an important role in the development of immunosensor, was evaluated at the level of its substrates. In this respect, TMB has been found to be the more specific and faster reacting substrate of HRP than ABTS. Therefore, the usage of TMB as a substrate in the HRP-based immunosensor designs was considered more appropriate than ABTS. This allows us to design a better immunosensor than the others.

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