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DEVELOPMENT OF AN ELECTROCHEMICAL IMMUNOSENSOR FOR THE DETECTION OF *E. COLI O157:H7*

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Biyoteknoloji Anabilim Dalı

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Ph.D. Thesis – Biotechnology
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ABSTRACT

An amperometric electrochemical immunosensor for the common food pathogen *Escherichia coli O157:H7* was developed. This novel immunosensor based on the PPy/AuNP/MWCNT/Chi hybrid bionanocomposite modified pencil graphite electrode (PGE). This hybrid bionanocomposite platform was modified with anti-*E. coli O157:H7* monoclonal antibody. The prepared bionanocomposite platform and immunosensor was characterized by using cyclic voltammetry (CV). Under the optimum conditions, the results have shown the order of the preferential selectivity of the method is gram negative pathogenic species *E. coli O157:H7*. Concentrations of *E. coli O157* from 3 x 10^1 to 3 x 10^7 cfu/mL could be detected. The detection limit was approximately 30 cfu/mL in PBS buffer. Briefly, we developed a high sensitive amperometric immunosensor for specific detection of *E. coli O157* contamination with the use of sandwich assay evaluated in this study offered a reliable means of quantification of the bacteria. For the applications in food quality and safety control, our immunosensor showed reproducibility and stability.

Keywords: MWCNT, Polypyrrole, Gold nano particles, Immunosensor, *E.coli O157:H7*.

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E. COLI O 157:H7 TESPİTİ İÇİN ELEKTROKİMYASAL İMMÜNOSENSÖR GELİŞTİRİLMESİ

Ahmet GÜNER

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ÖZ

Yaygın olarak bilinen ve tehlikeli olan gıda patojenlerinden *Escherichia coli* O157:H7 bakterisini tespit edebilen amperometrik elektrokimyasal bir immünosensör geliştirildi. Yeni bir yöntem olan bu immünosensör kalem ucu grafit elektrot üzerine hibrit biyonanokompozit PPy/AuNP/MWCNT/Chi ile modifiye edilme esasına dayanmaktadır. Bu hibrit kompozit platform üzerine *E. coli O157:H7* bakterisine özgü bir monoklonal antikor yerleştirildi. Bu şekilde hazırlanan platform CV (Cyclic Voltammetry) ile karakterize edildi. Uygun koşullar altında elde edilen sonuçlarda gram negatif ve patojen olan E. coli O157:H7 suşu bu metoda göre tespit edilebildi. Tespit edilen bakteri sayı aralığı ise 3 x 10^1 - 3 x 10^7 cfu/mL dir. PBS tampon çözeltisinde tespit edilme alt limiti ise 30 cfu/mL dir. Özet olarak, geliştirmiş olduğumuz bu amperometrik immünosensör; yüksek hassaslığının yanı sıra güvenilir, sadece *E.coli O157:H7* suşuna özgü bir sensör olduğu ve bu haliyle de gıda kalite ve güven kontrol çalışmalarında tercih edilebileceğini göstermiştir.

Anahtar Kelimeler: MWCNT, Polipirol, Altın nano parçacık, İmmünosensör, *E.coli O157:H7.*

DEDICATION

To my wife and my parents

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LIST OF SYMBOLS AND ABBREVIATIONS

AuNPs	Gold nanoparticles
BSA	Bovine Serum Albumin
CDC	Center for Disease Control and Prevention
CFU	Colony Forming Unit
CHIT	Chitosan
CNT	Carbon nanotube
CV	Cyclic Voltammetry
ELISA	Enzyme Linked Sorbent Assay
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
HHS	Health and Human Service
HRP	Horse radish peroxidase
mAb	Monoclonal Antibody
MWCNT	Multi walled carbon nanotube
NIAID	National Institute of Allergy and Infectious Diseases
pAb	Polyclonal Antibody
PBS	Phosphate Buffer Saline
PGE	Pencil Graphite Electrode
PPy	Polypyrrole
RT	Room Temperature
SPCE	Screen Printed Carbon Electrode
STEC	Shiga-toxin Escherichia coli
SWCNT	Single walled carbon nanotube
TSA	Tryptic Soy Agar
WHO	World Health Organization

CHAPTER 1

1.1 *INTRODUCTION*

This chapter will introduce the background knowledge about the problems with foodborne pathogens and overview of detection of foodborne pathogens. Objective of the thesis has also been addressed.

1.2 *PROBLEMS WITH FOODBORNE AND DISEASES*

One of the major challenges to public health concern is the continual outbreaks of pathogens in the food industry and threatening millions of lives annually in the worldwide (Lu and Jun 2012, Viswanathan, Rani et al. 2012). Approximately 48 million illness, 128,000 hospitalizations, and 3000 deaths related to the foodborne diseases in USA each year reported from Center for Disease Control and Prevention (CDC, www.cdc.gov/foodborneburden). The estimation number of illnesses annually because of the known pathogens, unspecified agents, and the total burden was indicated in Table 1.1.

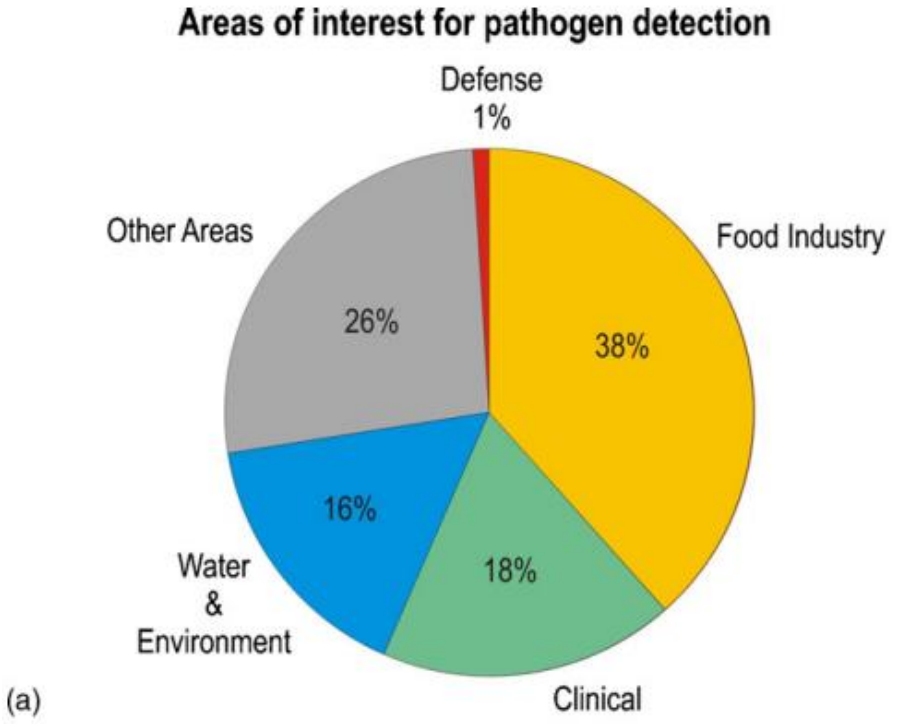
Table 1.1 Estimated annual number of domestically acquired foodborne illnesses, hospitalizations, and deaths in United States.

Foodborne agents	Estimated annual number of illnesses (90% credible interval)	%	Estimated annual number of hospitalization (90% credible interval)	%	Estimated annual number of death (90% credible interval)	%
31 known pathogens	9.4 million (6.6 – 12.7 million)	20	55,961 (39,534 – 75,741)	44	1,351 (712 – 2,268)	44
Unspecified agents	38.4 million (19.8 – 61.2 million)	80	71,878 (9,924 – 157,340)	56	1,686 (369 – 3,338)	56
Total	47.8 million (28.7 – 71.1 million)	100	127,839 (62,529 – 215,562)	100	3,037 (1,492 – 4,983)	100

Table 1.2 Top five pathogens causing domestically acquired foodborne illnesses resulting in hospitalization.

Pathogen	Estimated annual number of hospitalizations	90% Credible interval	%
Salmonella, nontyphoidal	19,336	8,545 – 37,490	35
Norovirus	14,663	8,097 – 23,323	26
Campylobacter spp.	8,463	4,300 – 15,227	15
Toxoplasma gondii	4,428	3,060 – 7,146	8
E.coli (STEC) O157	2,138	549 – 4,614	4
Subtotal			88

Among the foodborne pathogens, *Salmonella enterica*, *Listeria monocytogenes* and Shiga-toxin producing *Escherichia coli* are responsible for the majority of recent outbreaks and hospitalizations (Ohk and Bhunia 2013). In 2010, it has been speculated in the United States, *E. coli O157:H7* and *Salmonella* pathogens alone have caused nearly cases of 1.4 million foodborne illness that caused $2.7 billion in associated medical costs, productivity losses, and costs of premature deaths (Yoon and Kim 2012). According to the CDC report, it is estimated 2,138 hospitalizations causing by *E. coli O157:H7* related to foodborne diseases happened in the USA each year. Therefore, many recent outbreaks both in Europe and Northern America have been associated with a strain of *E. coli* which has been identified among the most common causes of diseases related to food safety in developing countries (WHO, 2008, http://www.cdc.gov/ecoli/2011/ecolio104/).



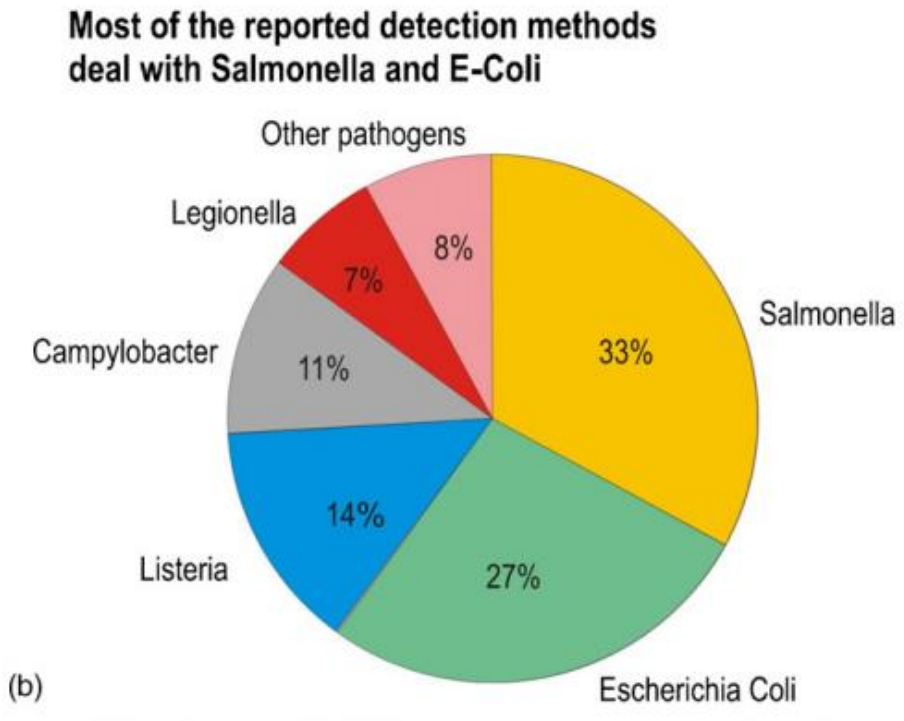


Figure 1.1 (a) Detection of pathogenic bacteria were distributed in industry applications. **(b)** Most of the reported detection methods deal with some known pathogens (Lazcka *et al*., 2007).

In developed countries, billions of dollars were lost in terms of medical care and lost productivity each year because of foodborne pathogens. Due to the fact that foodborne outbreaks in a lot of countries occurred because of the contaminated with pathogenic bacteria, chemicals and toxins. Thus, the safety of food has strikingly improved overall. On the other hand, some dangerous pathogens could be used to contaminate the water supplies and food in order to weaken hundred thousands of people for disrupting the economy and growing the anxiety by the terrorists. Enhancing the potential for outbreaks and health risks posed by foodborne pathogens are of major concern to all governments due to the trading of contaminated food among the countries. Henceforth, federal governments take some precautions for continuous outbreaks of illness and retracts of food products. FSIS, FDA, HHS, EPA are the main services that are taking major responsibilities for food regulating, food safety and developing novel inspection methods (Yang and Bashir 2008).

1.3 *Escherichia coli O157:H7*

Due to continued outbreaks and a major public health concern are the foodborne pathogens which is the most common and responsible for the majority of recent outbreaks and hospitalizations is shiga-toxin producing *E. coli* (Ohk and Bhunia 2013). This pathogen is a kind of potential marker used in the intestinal tracts of humans and warm-blooded animals (Li, Fang et al. 2013). A low number of *E. coli O157:H7* is adequate to cause severe sicknesses. The symptoms of infection with *E. coli O157* such as abdominal cramps, nausea, vomiting, bloody diarrhea, headache, and (in 2-7% of cases) life-threatening hemolytic uremic syndrome are characterized by kidney failure and hemolytic anemia (Barreiros dos Santos, Agusil et al. 2013, Dou, Tang et al. 2013, Wang, Ping et al. 2013, Chen, Yin et al. 2014). This pathogenic bacterium is a fecal contaminant and generally found in raw or undercooked meat, unpasteurized milk, unwashed product and sewage-tainted waters.

FDA reported the infectious dose of *E. coli O157:H7* was between 10 to 100 cells in 2009. Even if the less number of this pathogen spreads to the food or water, it will threat the human health obviously. Thus, it is not acceptable to be available at any quantity in food or water intended for human use. According to WHO reports, any strains of *E. coli* found in water supplies or food, it means that the evidence of recent fecal

contamination. Therefore, it should be investigated in terms of safety and quality control for public distribution. Moreover, WHO also stated that there must be no *E. coli* detectable in any 100 mL water sample intended for drinking or for public distribution (WHO 2006).

Consequently, this pathogen can cause incredibly huge health care costs and product recalls. Thus, a novel, rapid and reliable method should be developed for *E. coli O157:H7* detection.

1.4 *OVERVIEW OF DETECTION OF FOODBORNE PATHOGENS*

The first control point in the prevention of diseases caused by foodborne pathogens is monitoring. Effective detection and investigation methods are requisite to control pathogens in food products and carrying vital importance in terms of the prevention and determination of problems associated with the health and safety (Yang and Bashir 2008, Velusamy, Arshak et al. 2010). In order to detect and identify the pathogens from food, the classical microbiological procedures have been a standard for nearly one century and continue to be a reliable standard for ensuring food safety (Velusamy, Arshak et al. 2010).

Conventional methods for the detection and identification of pathogen are mostly depend on culture, microscopic, biochemical, immunological and genetic techniques (Mura, Greppi et al. 2012). However, these traditional methods for the detection of foodborne pathogens have disadvantages in various extents, owing to detection limit, detection rate, and associated cost, time-consuming, labor-intensive protocol and sensitivity (Lu and Jun 2012, Safavieh, Ahmed et al. 2012, Tawil, Sacher et al. 2012, Sharma and Mutharasan 2013). Therefore, they are not practical for rapid point of care diagnostics suitable for modern food quality assurance to make a timely response to possible risks (Yang and Bashir 2008).

In recent years, plenty of novel detection methods have been advertised that are exhibiting excellent sensitivity, reproducibility and capable of detecting pathogens in near-real-time (Yoon and Kim 2012). Among these methods biosensor technologies

have been used as potential alternatives to the standard methods because of these facilities (Afonso, Perez-Lopez et al. 2013).

Due to the specific antibody-antigen interactions, electrochemical immunosensors were pretty much preferred for the last decades. The reason for preference of this immunosensors are simple, sensitive, fast, selective, and cost-effective measurements with specific immunoassay procedures for detecting the pathogenic bacteria (Wang, Ping et al. 2013). In the future, Immunosensors should play a greater role in clinical and environmental analysis especially for food industry (Xie, Chen et al. 2011, Chen, Yin et al. 2014).

According to Yang and Bashir, they claimed that an ideal and a useful rapid method should be able to detect even if a single pathogenic microorganism present in the food. For instance, as low as 10 *E. coli O157:H7* cells represent the infectious dose and the existing coliform standard for *E. coli* in water is 4 cells/100 mL in food is 1 cell per 25 g. However, as they have mentioned about an ideal rapid and automated system that answers all the necessities still does not fabricate (Yang and Bashir 2008).

Eventually, it is necessary to upgrade new methods for identification of emerging hazard to food safety in time with the aim of preventing these hazards from becoming real risks and causing incidences.

1.5 *OBJECTIVE OF THE THESIS*

The ultimate goal of this thesis is to develop an amperometric electrochemical immunosensor for the measurement of common food pathogenic bacterium namely *Escherichia coli* O157:H7.

CHAPTER 2

LITERATURE SURVEY ON ELECTROCHEMICAL IMMUNOSENSOR

2.1 *IMMUNOSENSORS IN FOODBORNE PATHOGEN DETECTION*

2.1.1 Introduction to Immunosensors

Immunosensors have been greatly studied and developed due to the high demand of clinical diagnostics, environmental control and drug discovery. The research in immunosensors is to explore an efficient and reliable transducer that can convert an immunological binding event into a measurable signal with high sensitivity, high selectivity, low cost and simple operation (Sadik, Aluoch et al. 2009, Velusamy, Arshak et al. 2010, Sharma and Mutharasan 2013, Chen, Yin et al. 2014). A large number of materials and detection techniques have been investigated to improve the sensor's performance. The emergence of nanotechnology has attracted increasing attention of researchers on the revolutionary enhancement effects of nanomaterials on immunosensors.

In general, nucleic acids, cell receptors, enzymes and microorganisms in addition antibodies or its fragments come together thereby they form the biosensors (Holford, Davis et al. 2012). Therefore, an immunosensor is made by two essential components integrated: a *bioreceptor,* which responds to the desired compound and a *transducer,* for generating a measureable signal proportional to the concentration of the analyte (Su, Jia et al. 2011, Thakur and Ragavan 2013). Various biological receptors including microorganisms, tissues, organelles, cells, enzymes, antibodies and nucleic acids have been used in the fabrication of immunosensors (Velusamy, Arshak et al. 2010, Su, Jia et al. 2011).

Based on the position of the transducer, there are three main types of immunosensors; the products of the reactions diffuse to the transducer causing an electrical signal; the products of the reactions are identified by a mediator that interacts with the transducer to generate the signal; the reaction itself is recognized by the transducer. The transducer can then be classified according to the mechanism of analyte recognition. According to the type of signal used for gathering info about the analyte can be identified as electrochemical, optical, and thermal biosensors (Chaki and Vijayamohanan 2002).

Over the last few years, depending on the electrode material, polypyrrole (PPy) has been extensively studied as an efficient matrix in immunosensors to enhance response time, sensitivity and versatility because of its good compatibility with biological molecules in neutral aqueous solutions. The PPy can also provide a 3D electrically conducting structure to connect the electron transfer from biomolecules such as the enzyme, to electrode (Raicopol et al., 2013).

The conductive polymers changes over several orders of amplitude in response to changes in pH and redox potential of their environment (Gerard et al., 2001). In one study, glassy carbon electrode was modified with PPy/Au nanocomposite through the electro-synthesis (Chen et al., 2007). According to the results, AuNPs were uniformly dispersed in the PPy nanocomposite film and the film showed an excessively microporous structure with polymer fibrils measuring diameters in a micrometer range. The formation mechanism was indicated that AuNPs exhibited great importance to form the pyrrole polymerization onto the electrode surface during electro-synthesis. This occurrence at the early deposition stage were conducted on AuNPs by the growth of PPy/Au film. In conclusion, PPy comprised the AuNPs and microporous form was produced and this incorporation of AuNPs in the PPy matrix significantly improved electronic conductivity, porous structure and electrochemical stability, especially in a neutral solution. Especially PPy/Au nanocomposite is an advanced material and excellent candidate for the biological sensors.

As can be understood from these studies, immunosensor applications play important roles on medical biosensor systems, quality control of food, agriculture and environmental and bioprocess monitoring. In addition to this, immunosensors are also

quite important because of their cheapness, portability, good selectivity and ability of measuring samples with minimal sample preparation (Pedrero, Campuzano et al. 2009, Arora, Sindhu et al. 2011).

2.1.2 Types of Immunosensors

Depending upon the working principle of immunosensors they are classified into different types. Some of the significant ones are explained below.

2.1.2.1 Electrochemical Immunosensor

Rapid, sensitive and selective detection of certain pathogenic bacteria from food in early determining is critical for the public human health. Recently, considerable attention has been paid to development of electrochemical immunosensors based on micro-fabrication, high specificity, sensitivity, selectivity, rapidity, ease of use, low limits of detection and sensitive detection inherent with biological binding and low cost (Liu and Lin 2007, Conroy, Hearty et al. 2009, Pedrero, Campuzano et al. 2009, Privett, Shin et al. 2010, Holford, Davis et al. 2012). Electrochemical immunosensors measure current potential changes due to interactions that occur at the sensor electrode and sample matrix interface (Sharma and Mutharasan 2013). These sensors are very sensitive and have been used to detect *Salmonella* and *E. coli O157:H7* in less than 90 min (Arora, Sindhu et al. 2011). Therefore, immunosensors provide an opportunity to gain new insight to develop simple and sensitive immunoassay apparatus in the detection of such foodborne pathogens.

Electrochemical immunosensors incorporate the high specificity of antibody-antigen interaction techniques with the low detection limits of contemporary electrochemical system. The crucial steps in designing of an immunosensor are the choice of the basis electrode and immobilization of the immunoreagent onto the electrode surface (Díaz-González et al., 2005).

To the point of Wang and coworkers view, an ideal immunosensor design should possess the ability to detect and quantify the antigens (antibodies), the capacity to transform the binding event without externally added reagents, and the ability to repeat the measurement on the same device, and the capacity to detect the specific binding of

the antigens (antibodies) in real samples. All of these specifications have been the main issues to pursue in developing immunosensors applied in various fields (Wang et al., 2008).

According to the detection principle and depending on the type of transducer used, electrochemical immunosensors are classified as amperometric, potentiometric and impedimetric immunosensors (Conroy, Hearty et al. 2009, Arora, Sindhu et al. 2011, Su, Jia et al. 2011, Holford, Davis et al. 2012).

2.1.2.1.1 Electrode Systems Used for Immunosensors Fabrication

To measure electrochemically in immunosensor procedure three electrodes systems are used including a working electrode, reference electrode and counter (auxiliary) electrodes. The working principle is the applying a constant potential between the working and reference electrodes. Thus, a redox reaction occurs and a current proportional to its concentration is measured between the working and counter electrodes (Wang, Quan et al. 2006). The electrodes transfer the electrons to the redox species as a result the current signal is produced at the surface. Meanwhile, for this immunosensor fabrication techniques there are variety of electrode materials used including platinum, gold, carbon, graphite etc. (Rao, Sharma et al. 2006).

In this case, and applied in this thesis, a pencil graphite electrode (PGE) was used in the development of an immunosensor and designed with a three electrodes system (working, reference and counter). The PGEs have been broadly used because they are economical (easy to fabricate in bulk and disposable), easy to handle, have high sensitivity and a miniaturized portable system.

2.1.2.1.2 Cyclic Voltammetry

One of the traditional method used in electrochemical reactions for acquiring generating qualitative information is Cyclic Voltammetry (CV). In order to measure the voltage under the control of a potentiostat, there are two different electrodes used *working* and *counter*. In this way the redox potentials were determined by the voltammogram during the electrochemical processes. The current density is then plotted against the

applied potential and the result is referred as cyclic voltammogram (Bard & Faulkner, 2001).

Due to the rapid location of redox potentials of the electroactive species, and convenient evaluation of the effect of media upon the redox process, it is usually the first experiment performed in an electrochemical study. Thus, it enables the electrode potential to be rapidly scanned in search of redox couples that are characterized from the potentials of peaks on the cyclic voltammogram. This alteration is because of the scan rate variation. The redox behavior over a wide potential range can be rapidly observed after the CV results represented in Fig 2.1 that illustrates the response of a reversible redox couple during a single potential cycle. The bulk solution only contains the reduced form of the redox couple (R) at the beginning of the experiment so there is no net conversion of R to O at this point, hence the potential is lower than the redox potential. As the redox potential is approached, there is a net anodic current which increases exponentially with potential until a peak is reached. At the anodic point, the redox potential is sufficiently positive which can convert R to O. Upon reversal of the scan, the current continues to decay until the potential nears the redox potential. At this point a net reduction of O to R occurs which causes a cathodic current and produced a peak shaped response (Bard & Faulkner, 2001; Wang, 2006; Zoski, 2007).

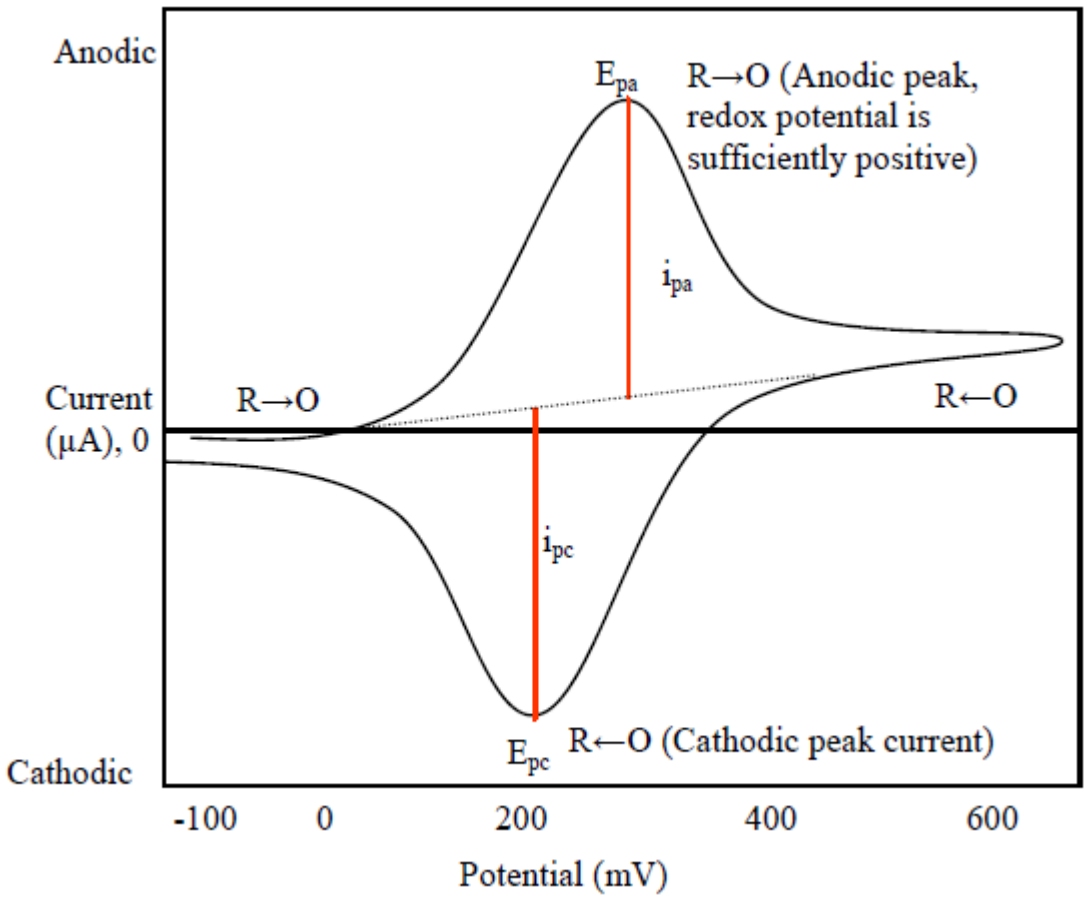


Figure 2.1 A typical cyclic voltammogram of species in solution for the important peak parameter. A reversible reaction of cathodic (c) and anodic (a), E_p refers to peak potential and i_p refers to peak current (Wang, Quan et al. 2006; Zoski, 2006).

The transmission of the information as an energy scan by means of the resulting voltammogram that is analogous to conventional spectrum. CV includes cycling the potential of an electrode specifically that is immersed in an unstirred solution, and measuring the resulting current. The potential of this working electrode is controlled versus a reference electrode.

2.1.2.2 Potentiometric Immunosensor

Potentiometric measurements are determined based on potential difference between the working electrode and the reference electrode. This potential signal exhibits concentration-dependent behavior (Su, Jia et al. 2011, Thakur and Ragavan 2013). Science, potentiometer generates a logarithmic concentration response the technique has a wide dynamic range that is quite sensitive to detect very low concentration changes (Velusamy, Arshak et al. 2010, Sharma and Mutharasan 2013). There are some potentiometric devices are commonly used such as pH electrodes, ion or gas selective electrodes (Thakur and Ragavan 2013).

These biosensors are generally used for the pathogen detection depending on pH changes or ion concentrations fluctuations in terms of portable and inexpensive (Arora, Sindhu et al. 2011). Potentiometric sensors are classified as ion-selective field effect transistors (ISFETs) and light-addressable potentiometric sensors (LAPs) (Sharma and Mutharasan 2013). ISFETs are useful for point of care and forensic applications in terms of high sensitivity, miniaturization and multichannel testing properties (Holford, Davis et al. 2012).

If we look at the literature what have been done about potentiometric immunosensors until now, Kumar and his colleagues developed a potentiometric biosensor associated with the pH electrode modified by permeant *P. aeruginosa* in order to selective and rapid detection of cephalosporin group of antibiotics. According to their results cephalosporin hydrolysis was accompanied by the production of protons near the pH electrode due to the enzyme activity of the microbial layer. In this case, the response occurs between the working electrode and the reference electrode resulting the change of electric potential difference (Kumar, Kundu et al. 2008). In another study, *E. coli* was detected from vegetable food by using the potentiometric biosensor based on LAPs.

2.1.2.3 Conductimetric Immunosensor

The changes in conductivity of the electrolyte between two electrodes depend on the conductimetric analysis. Thus, the migration of ion induced by an electrical field is the result of conductivity which is the dissociation process of the dissolved substance in an electrolyte into ions. This ion movement is influenced by the application a potential

difference to the electrode. As it is demonstrated in Fig 2.7, those with negative charge more towards anodes, while positively charged ones move towards the cathodes. As a result, the conductivity of the electrolyte solution is based on the concentration of the ion and the mobility. However, conductimetric measurement is rarely used in immunosensor set-up in contrast to potentiometric and amperometric measurements. This is because of the nonspecific binding problems on the transducer surface and poor signal ratio during the measurement (Nicole & Sergei, 2008).

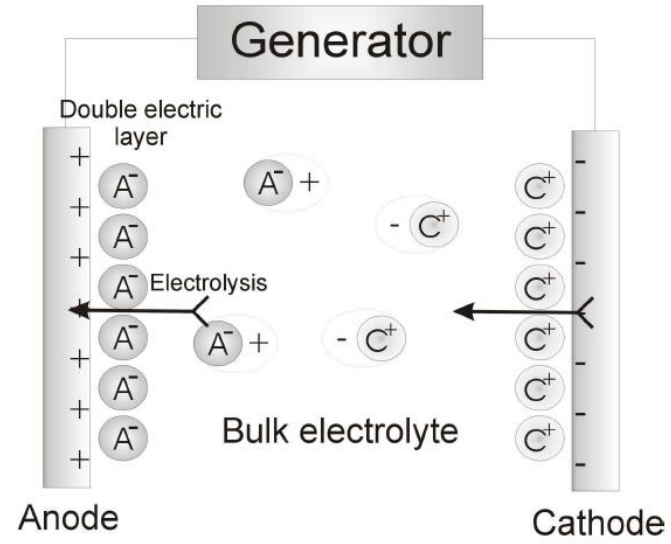


Figure 2.2 The current measurement in conductimetric immunosensors is based on changing of conductivity of the electrolyte which is caused by the ion movement toward the electrode (Nicole & Sergei, 2008).

Table 2.1 briefly summarizes the comparison of the different electrochemical transducers used in electrochemical immunosensors.

Table 2.1 Advantages and disadvantages of three transducers used in electrochemical immunosensors (Cunningham *et al*., 1998).

Type of energy transduction	*Advantages*	*Disadvantages*
Amperometry	✓ Wide variety of biochemical redox ✓ Mechanism as basic for signal generation ✓ Easily miniaturized ✓ Easily produced in bulk (screen printed electrode) ✓ Good dynamic ranges, controlled by sensor surface thickness ✓ Relatively good sensitivity	✓ Required a reference electrode ✓ Multiple step of immobilization of reagent is required to increased sensitivity
Potentiometry	✓ Ion Selective Electrode translation is easy ✓ Easily miniaturized	✓ Required a reference electrode ✓ Limited linear range ✓ Often pH sensitive
Conductiometry	✓ Easy to fabricate ✓ No Reference required ✓ Low frequency/amplitude source	✓ Non-selective unless use array format

2.1.2.4 Amperometric immunosensor

These immunosensors are able to detect by applying a constant potential between the working electrode and the reference electrode and the current signal associated with the reduction or oxidation of an electroactive species involved in the recognition process is recorded and correlated with the concretion of target compounds (Arora, Sindhu et al. 2011, Su, Jia et al. 2011, Shinde, Fernandes et al. 2012). In the amperometric detection, the current signal is generated during the oxidation and reduction reactions between the working electrode and reference electrode. While the analyte binds to the working electrode, no specific binding occurs in reference electrode (Conroy, Hearty et al. 2009). In most cases, amperometric immunosensors generally based on *horseradish peroxidase* and *alkaline phosphatase* enzymes that catalytically converts electrochemically noactive analytes into electrochemically active products (Arora, Sindhu et al. 2011).

The most common amperometric measurements are performed in an analyte in a three-electrode arrangement. The current is measured at the site of *the working electrode* in this system. A potential is applied to this electrode relative to a second *reference electrode*, also in contact with the test solution, in order to generate a potential at the interface between the working electrode and the solution. When necessary, *the counter electrode* can supply current to the test solution and the electrode was monitored by the

reference electrode (Daniels and Pourmand 2007). This situation is the part of a feedback system.

An amperometric immunosensor have a great impact among the biosensor technology in terms of miniaturization of electrochemical systems, reducing the sample and reagent volumes and response time due to rapid diffusion as well as its portability and cheapness. Therefore, the usage of this miniaturized electrodes in micrometer to millimeter sized have been increased day by day.

Generally screen-printed electrodes are formed semi-conductive ink with a specific shape, size and constant thickness are used in sensing chips. They are mass-produced, inexpensive, and often designed to be disposable, which decreases the possibility of electrode fouling, cross-contamination between samples, and irreproducibility (Palchetti and Mascini 2008). Screen-printed carbon (Obuchowska 2008, Shabani, Zourob et al. 2008) and gold (Susmel, Guilbault et al. 2003, Escamilla-Gomez, Campuzano et al. 2009, Gamella, Campuzano et al. 2009) electrodes were applied to impedance-based detection of *E. coli* and other bacterial targets. Also, conductive nanoparticles are sometimes coated onto screen printed electrodes in electrochemical biosensor assays, for signal transduction and amplification purposes. Gold is the most common choice in conductive nanoparticle for facilitating electron transfer at electrode surfaces (Lin, Chen et al. 2008). In addition to gold, other nanoparticles employed for electrochemical biosensor enhancement include silver, platinum, copper, and silicon dioxide (Wang and Jin 2003).

Lin et al. (2008) fabricated a disposable amperometric immunosensing strip for rapid detection of *E. coli O157:H7*. Lin and his coworkers designed their system for the rapid, sensitive detection of bacterial cells utilizing AuNP and ferrocenedicarboxylic acid (FeDC)-modified, disposable screen printed carbon electrodes (SPCEs) (Fig 2.2). As a result of their study, they reported that they could detect approximately 50 CFU of *E. coli O157:H7* in milk samples in 1 h.

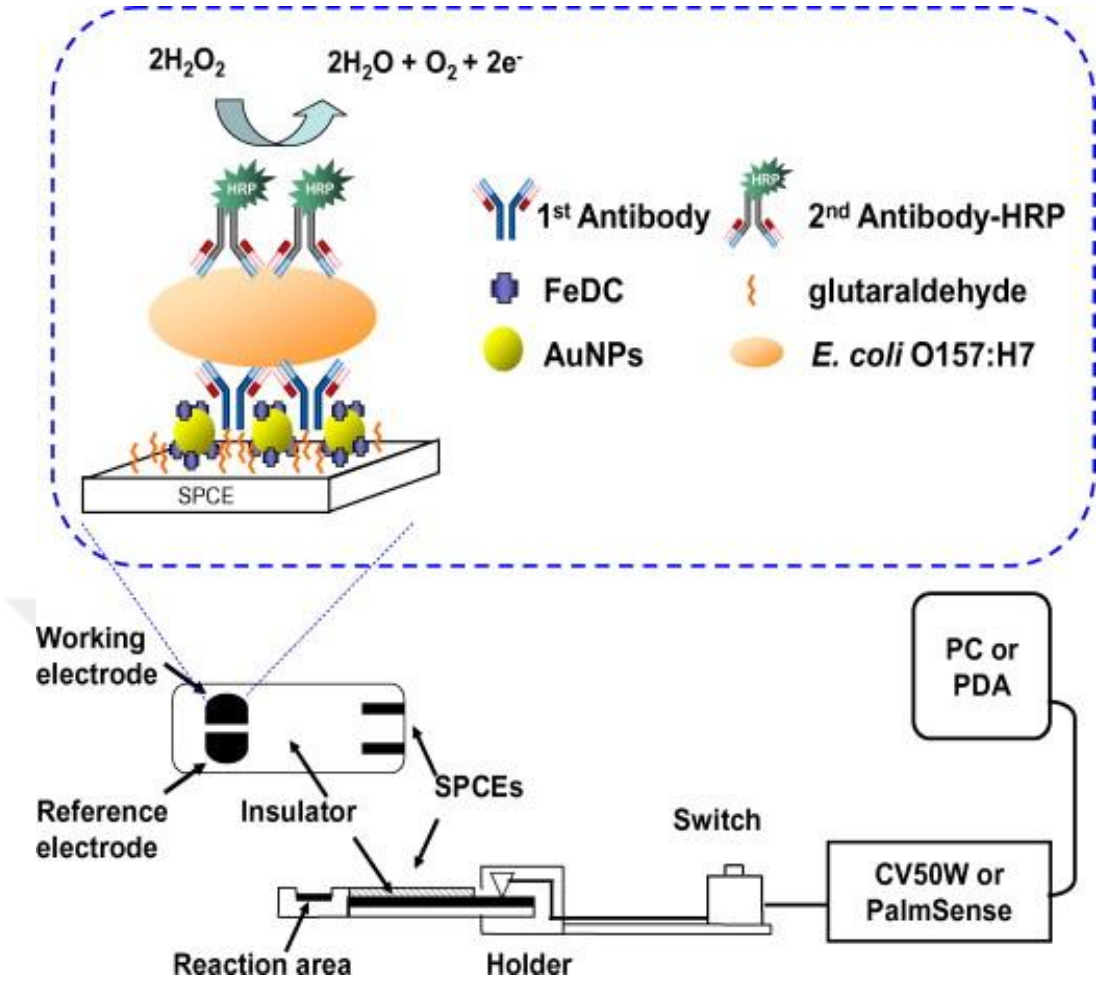


Figure 2.3 Schematic representation of the apparatus and immunosensing processes of the AuNPs/FeDC–SPCE immunosensor system for *E. coli O157:H7* detection. The AuNPs and FeDC were modified on the working electrode and the detection model was shown in the top panel.

In one study in 2012 Viswanathan and his colleagues developed a disposable electrochemical immunosensor for detection of *E. coli O157:H7*, *Campylobacter* and *Salmonella* simultaneously from the milk samples. They detected these bacteria by using screen printed electrode immunosensor modified with multiwalled carbon nanotube-polyallylamine (MWCNT-PAH/SPE) and nanocrystal (CuS, PbS and CdS) antibody conjugates (Fig 2.3). According to their results, they suggested that this immunosensor could be applied to monitor for food quality control. However, they reported that it will be necessary to perform more experiments with a greater number of samples in order to validate sensitivity and specificity (Viswanathan, Rani et al. 2012).

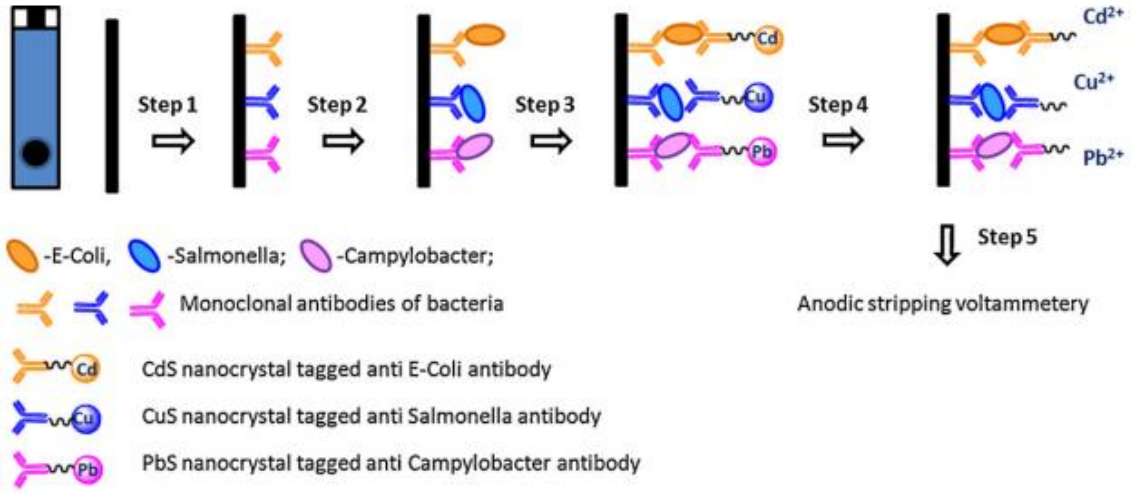


Figure 2.4 Schematic representation of multiplexed detection of pathogens using NC antibody conjugates and MWCNT-PAH/SPE. Antibody immobilization (Step 1); Immune-capture (Step 2); Immune-binding (Step 3); Dissolution of metal ions from NC (Step 4); SWSV analysis (Step 5) (Viswanathan, Rani et al. 2012).

Dou and his coworkers developed a multiplexed electrochemical immunosensor simultaneously detected *E. coli O157:H7* and *E. sakazakii* (Fig 2.4). They used four channel screen printed carbon electrode modified with MWCNT/SA/CMC. Their antibodies labeled with HRP in order to enhance the direct electrochemical responses. They reported that their results showed acceptable linear ranges and accuracy for detection of pathogen an exhibited acceptable specificity, reproducibility and long-term stability (Dou, Tang et al. 2013).

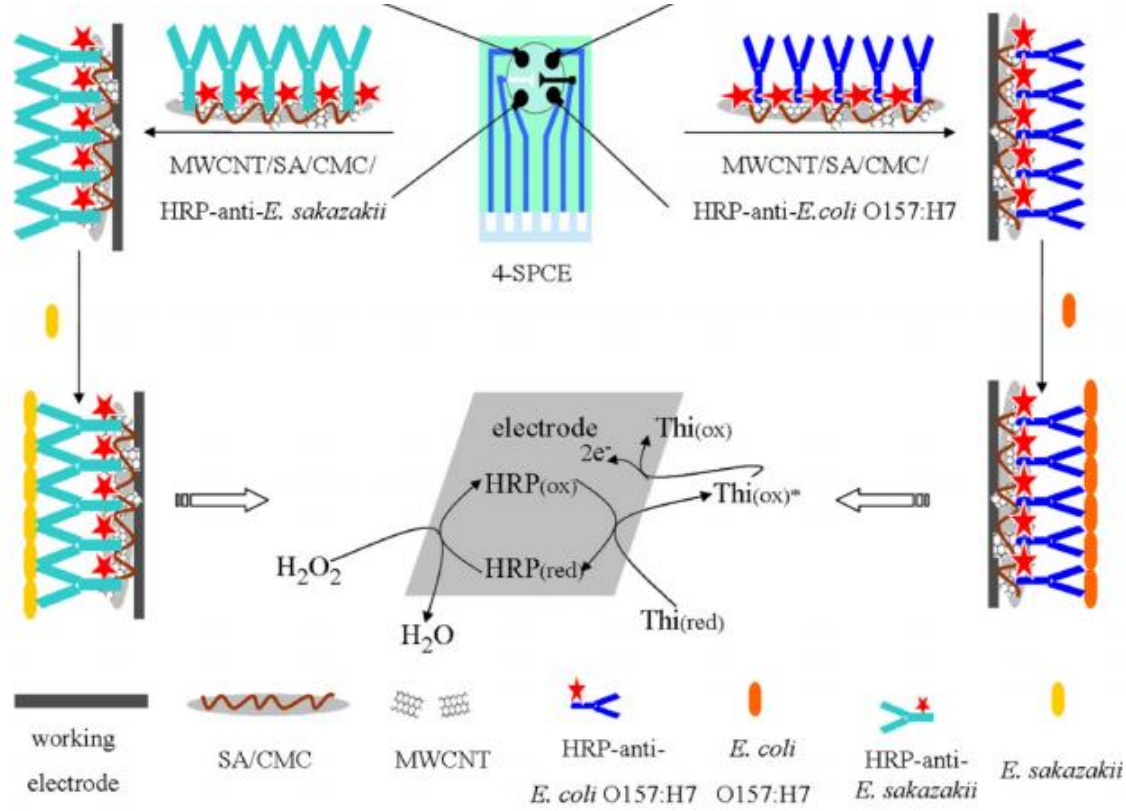


Figure 2.5 Schematic illustration of immunoelectrode fabrication for the determination of *E. sakazakii* and *E. coli O157:H7*. Thi(ox) and Thi(red) are the oxidized and reduced forms of thionine, respectively (Dou, Tang et al. 2013).

In another study Li and his coworkers developed a novel, label-free amperometric immunosensor for the rapid detection of heat-killed *E. coli O157:H7* used a chitosan-multi walled carbon nanotubes-SiO_2/thionine nanocomposite material in terms of abundant $–NH_2$ groups, which provide strong adsorption ability to AuNPs for electrochemical biosensing platforms (Fig 2.5). According to their results, the sensitivity of the detection limit was 250 CFU/mL E. coli O157:H7 in PBS and they successfully tested *E. coli* in both milk and water samples (Li, Cheng et al. 2012). The next year Li and his group designed a biocompatible and sensitive amperometric immunosensor for heat-killed *E. coli O157:H7* detection based on Au-SiO_2 embedded fullerene/ferrocene and thiolated chitosan (C^{60}/Fc/CHI–SH) (Fig 2.6). For signal amplification, they used glucose oxidase loaded Pt nano-chains as tracing tag label signal antibodies. Due to sandwich-type, biotin–avidin system and synergetic catalytic of Pt nano-chains, they have improved sensitivity of the immunosensor for *E. coli O157:H7* with a detection limit of

15 CFU/mL, a value below the commonly accepted concentration threshold in clinical diagnosis (Li, Fang et al. 2013).

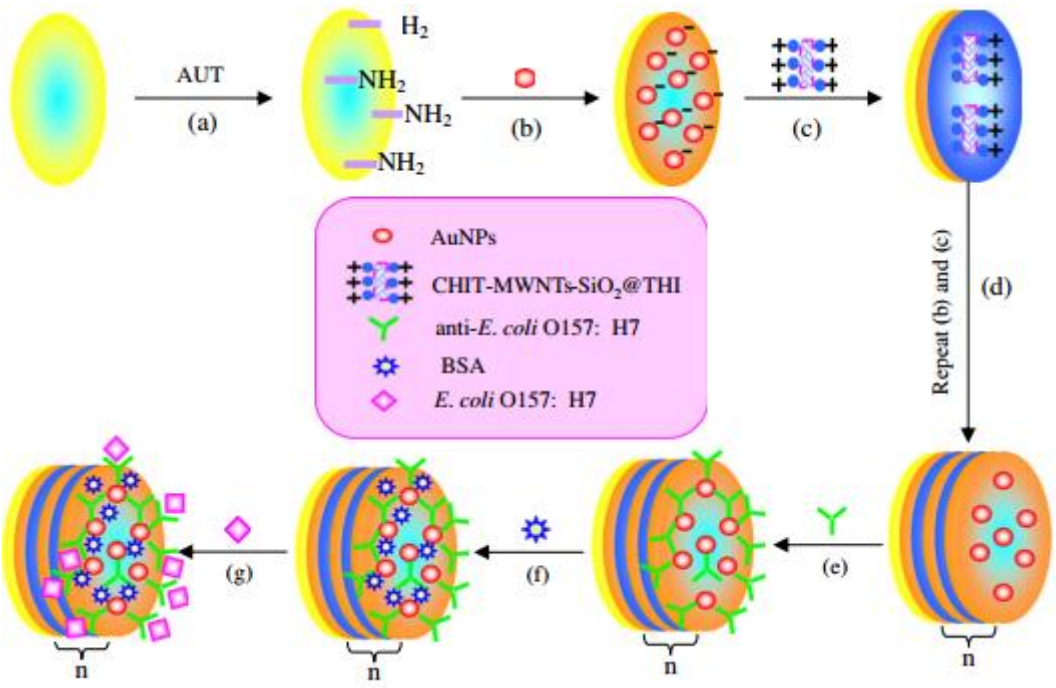


Figure 2.6 Schematic illustration of the preparation process of the immunosensor (Li, Cheng et al. 2012).

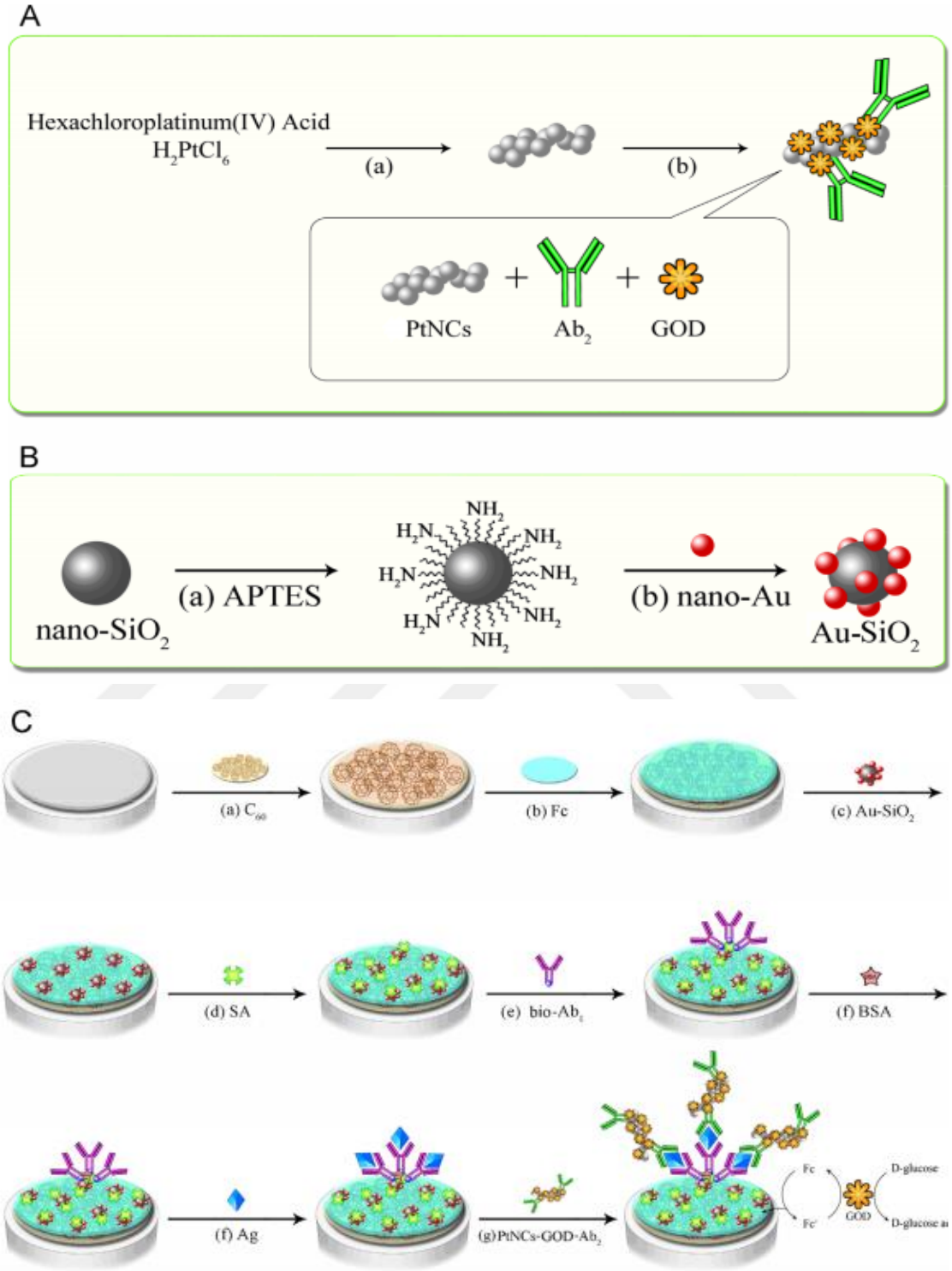


Figure 2.7 Scheme for (A) preparation of PtNCs–GOD-Ab_2 bioconjugates; (B) preparation of Au-SiO_2 nanocomposites; and (C) the fabrication of the electrochemical immunosensor and amplified detection.

2.1.3 Nanomaterials Used in Immunosensor

In recent years, various types of nanomaterials have been enlisted as labels to construct electrochemical immunosensors including chitosan, gold nanoparticles, polypyrrole, ferrocene and carbon nanotubes, have been intensively investigated, due to their chemical and physical features.

Chitosan, which has good adhesion, good film-forming ability and high mechanical properties, has been extensively used in many electrochemical biosensors. Chitosan is a non-toxic polysaccharide that possesses biocompatible and biodegradable properties. In addition to these features it is abundant in terms of -NH_2 and -OH functional groups, thus, it can be used to prepare CNTs/Chi composite for enhancing the application of CNTs (Madihally et al., 1990). CNTs/Chi composite possesses excellent mechanical, biological and photoelectric features for its unique structure and is widely applied in fabrication of electrochemical biosensors [Najeeb et al., 2011, Zhao et al., 2012).

AuNPs have been commonly preferred for immunosensor fabrication due to its excellent electrical conductivity, large specific surface area and strong ability to adsorb proteins with preserved bioactivity (Liu et al., 2012). In some recent studies showed that AuNPs provide an environment similar to the native system of the immobilized biomolecules because they are unstable and aggregate easily in the solution or on the solid surface, which effectively retains their biological function (Xiang et al., 2015).

Due to its long term environmental stability (Kausaite-Minkstimiene et al., 2011), biocompatibility (Ferraz et al., 2012) and good chemical and thermal stability (Mehdinia et al., 2012) under various experimental conditions and biodegradation in composition with biodegradable polymers (Boutry et al., 2012), PPy has been used to construct electrochemical biosensors (Deepa et al., 2008; Zhang et al., 2009; Pertines et al., 2011). PPy is often applied in the design of biosensors based on immobilized enzymes (Banu et al., 2008) or antibodies (ElKaoutit et al., 2011). To enhance its functions PPy can be easily modified by covalently attached redox groups (Li et al., 2012) and proteins (Chen et al., 2010). Briefly, PPy is an efficient matrix and electrode material for immunosensors because of good compatibility with biological molecules and the ease of immobilization of various biologically active compounds and also great potential to enhance response time, sensitivity and versatility (Ramanaviciene et al., 2002, Li et al., 2005).

In combination with electronic conductivity of the conducting polymer and functionalized PPy dendrimers under ferrocene (Fc) redox properties play an important role in electron transferring and developing sensing behavior (Saleem et al., 2015). Covalent functionalization is a key factor for conducting polymers because it averts the mediator leakage by attachment of the Fc-based mediators on top of polymeric films. Therefore, the leakage problem from the electrode surface was eliminated by the help of Fc-functionalized PPy derivatives connected covalently (Şenel, 2011). Electrochemical polymerization is a better solution for producing PPy films in immunosensor development. Applications in biosensors and ease of depositing from nonaqueous electrolytes are quite advantageous properties of functionalized PPy. Thus, derivatives of Fc-based PPy showed well adherent film formation by using potential sweeping in redox electrochemistry. In biosensor applications, Fc-based PPy enhance the redox properties and in this case, they provide better electron transfer and better electrochemical for biosensors (Palomera et al, 2011). Consequently, modified electrodes comprising Fc-based PPy films have gained considerable attention in rapid heterogeneous electron transfer rates.

In the interest of developing biosensors, carbon nanotubes are quite new nanomaterials and carry the importance for CNT applications in electrochemistry. It has been indicated that the prominent electrochemical features of CNTs as a result of their richness in surface structure and chemistry. This provides enhancing electron transfer rates, increasing signal currents and lowering over-potentials. There are two different cylindrical structures of carbon allotropes made of graphene sheets such as single-walled (0.4-1.5 nm diameter) and multi-walled (2-100 nm diameter) carbon nanotubes. (Figure 3.1).

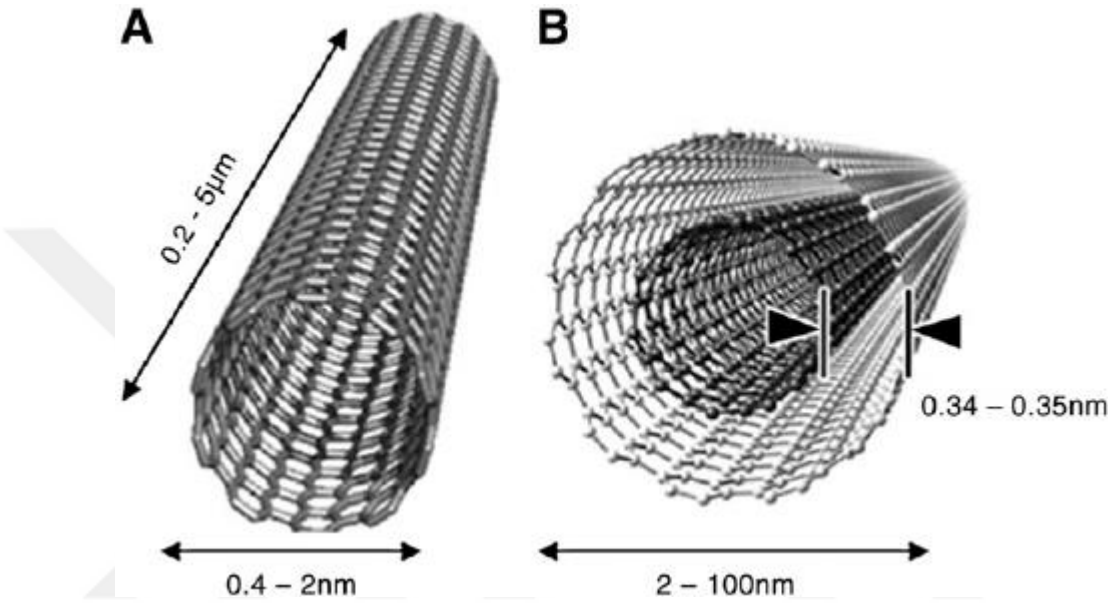


Figure 2.8 Conceptual diagram of (A) single-walled carbon nanotube and (B) multi-walled carbon nanotube - Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/42588327_fig1 [accessed Feb 1, 2016].

CHAPTER 3

MATERIALS AND METHODS

The procedures, materials and methods of this work have been mentioned in this chapter to analyze the detection of *E. coli* O157 by an amperometric immunosensor. In addition to this, many parameters of this immunosensor were evaluated.

3.1 *CHEMICALLY MODIFIED ELECTRODES*

Frequently, the modification of the original surfaces of electrodes is appropriate in order to use preferred new properties (Alkire *et al.*, 2009). In terms of basic electrochemical studies as well as in the design of electrochemical devices for electrochemical sensing, electro-synthesis, corrosion protection, electrochromic displays, energy conversion and storage, and molecular electronics, this approach is very useful.

The chemically modified and designed electrodes which have electro-catalytic features increase the detection signal and also lower the over-potential essential for the detection. The slow rate of electron transfer of unmodified electrode is accelerated by the electro-catalysis at the modified electrode with the same potential. The immobilization of a redox mediator (catalyst) on the surface of electrode prepares the electro-catalytic modified electrodes. The electro-catalysis at such electrodes is accomplished by charge mediation, which is necessary for the difference in the formal potentials of the catalyst and analyte be thermodynamically convenient. Either covalently attaching mediators directly to the electrode surface or by immobilizing them in thin polymeric films cast on the electrode surface have been prepared various electro-catalytic electrodes.

3.2 *REAGENTS AND MATERIALS*

All reagents were of analytical grade and were used without further purification. Hydrogen peroxide (H_2O_2, 30%, w/w) was obtained from *Sigma Aldrich* and solutions were freshly prepared before being used. Phosphate buffer solutions with various pH values were prepared by mixing standard stock solutions of 0.10 M Na_2HPO_4 and 0.10 M NaH_2PO_4 and adjusting the pH with 0.1 M H_3PO_4 or NaOH from *Merck*, (Germany). All solutions were prepared using Milli-Q water (resistivity 18 MΩ cm^{-1}). $K_3[Fe(CN)_6]$, $K_4[Fe(CN)_6].3H_2O$, Chlroauric acid ($HAuCl_4$), glutaraldehyde, glycerol, glycine, sodium chloride (NaCl), bovine serum albumin (BSA) obtained from *Sigma Aldrich*, (USA). 1-(2-carboxyethyl) pyrrole was obtained by hydrolysis of 1-(2-cyanoethyl) pyrrole (Py-CN) according to literature (Wolowacz et al., 1992). *E. coli O157:H7* was purchased from ATCC 43895. Monoclonal anti-*E. coli O157:H7* antibody (ab20976) and horseradish peroxidase-conjugated polyclonal anti-*E. coli O157:H7* antibodies (ab20425) were purchased from *Abcam*, (Cambridge, UK). The preparation of the buffers was explained by Lin et al. (2008), but with minor modifications.

3.3 *APPARATUS*

The cyclic voltammetry (CV) and amperometric measurements were performed using Ivium Compactstat and CHI Model 842B electrochemical Potentiostat/Galvanostat. Working electrode was a PGE purchased as pencil lead from a local bookstore. A platinum wire counter electrode (0.4 mm diameter, purchased from *EMC-Technologies*, Turkey) and an Ag/AgCl-saturated KCl reference electrode were used as auxiliary and reference electrodes, respectively. InoLab pH-meter (Model 7110) with a glass electrode (Corning) was used to measure the solutions pH.

3.4 *SYNTHESIS OF PYRROLE BRANCHED-CHITOSAN*

Pyrrole branched chitosan was synthesized according to our previous study (Şenel, 2015). Methanol was added to 1% (w/v) chitosan dissolved in acetic acid solution in order to obtain 85:100 ml methanol and chitosan solution respectively. The addition of PyPA to the chitosan solution in the ratio of 0.54 mol/mol glucosamine residue of chitosan was followed by dropping 15 mL of 0.07% (w/v) EDC dissolved in methanol when stirred at room temperature. In the current study, equal moles of EDC and PyPA were used. At

the end of 24 h, the reaction mixture was transferred into 200 ml of 7/3 (v/v) methanol/ammonia solution while being stirred. The primitive product was then filtered and washed with distilled water, methanol, and ether respectively, followed by being dried under vacuum for 24 h at room temperature.

3.5 *ELECTRODE MODIFICATION*

PGEs were washed with absolute ethanol first, then rinsed with distilled water. After drying, in order to modify the electrodes, spread 10 µL of the Chi-PPy-Fc-MWCNT solution with $HAuCl_4$ over the PGE surface, and then set it at the room temperature to evaporate the solvent. Then a volume of 10 µL of 2.5 mM glutaraldehyde as a crosslinking agent was dropped onto the working electrode area in order antibodies to bind, incubated for 1 h at 4°C and washed twice with PBS buffer. Finally, on the surface of the electrode, a film will form, which can be visually observed. All these procedures were summarized in Fig 3.2.

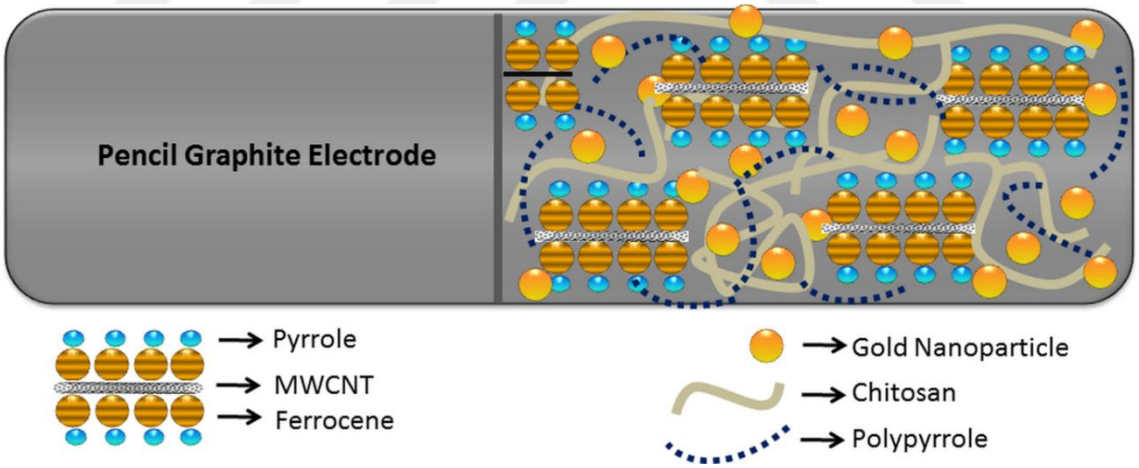


Figure 3.1 Schematic illustration of the preparation process of the modified pencil graphite electrode.

Amperometric detection allows for miniaturization and portability of the immunoassay system, since the equipment that would be required is readily available. A sandwich-scheme can be utilized, which is illustrated in Fig 3.2. In this figure, antibodies specific to *E. coli O157* are immobilized on the surface of the PGE. When bacteria

entrapped by the immobilized antibodies, a second set of antibodies (immunoconjugate), which are labeled with HRP were added to be detected.

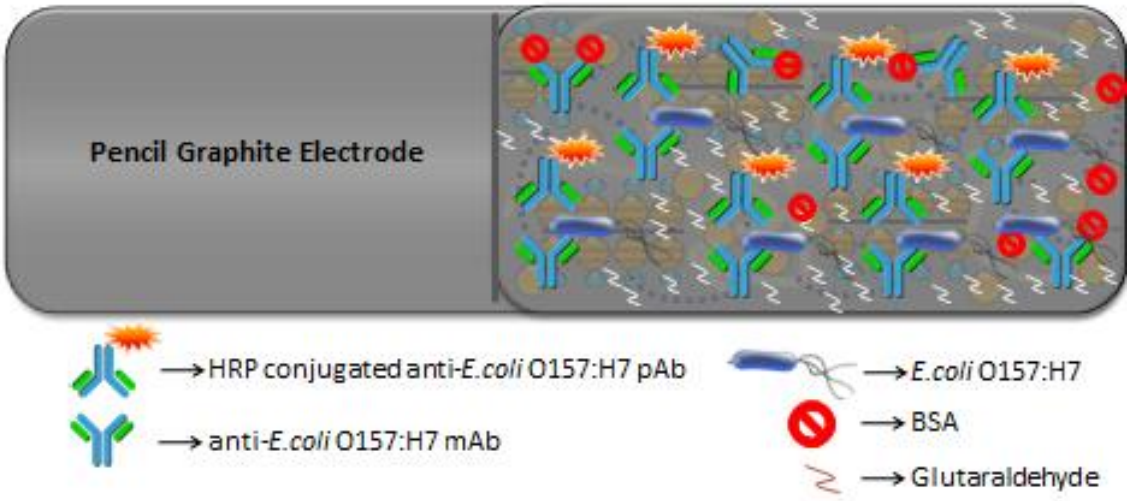


Figure 3.2 Schematic illustration of the apparatus and immunosensing processes of the *PPy/AuNP/Fc/MWCNT@Chi* – PGE immunosensor system for *E. coli O157:H7* detection.

3.6 *PREPARATION OF MICROBIAL SAMPLE*

The bacterial cultures, including *E. coli O157:H7* (ATCC 43895) strain was used in this study obtained from American Type Culture Collection (ATCC). The pure cultures were grown in Tryptic Soy Agar (TSA, BD Difco^{TM}) at 37^{o}C for 24 h before use. A conventional spread plating method was used for bacterial counts.

3.7 *IMMUNOASSAY PROCEDURE*

Each working electrode of the Chi-PPy with $HAuCl_4$ was incubated with 10 µL of the monoclonal anti-*E. coli O157* antibodies (10 µg/mL) for 15 min at 37^{o}C and blocked with 10 µL blocking buffer 2% BSA for 60 min at 4^{o}C. Between each step, each electrode was washed twice with PBS buffer (pH 7.0). A volume of 10 µL bacterial sample was applied to the immunosensing site on the working electrode and incubated for 15 min at 37^{o}C. A volume of 10 µL of HRP-conjugated polyclonal anti-*E. coli O157* antibody (5 µg/mL) was applied to the immunosensing site and incubated for 15 min at 37^{o}C. The

electrodes were then washed twice with PBS buffer to remove non-specific binding between antigens and antibodies.

All the experiments were completed at room temperature. Amperometric studies were measured in stirred solutions under the appropriate potential. Once prepared, the *PPy/AuNP/Fc/MWCNT@Chi* electrodes with bacteria were immersed in 10 mL of 0.1M PBS solution at pH 7.0 and the amperometric response to the addition of known amount of H_2O_2 solution was recorded. All these procedures were summarized in Fig 3.3.

3.8 *EXPERIMENTAL MEASUREMENTS*

Newly designed electrodes were analyzed by Cyclic voltammogram (CV) in a conventional electrochemical cell containing a three-electrode arrangement. The CVs were performed 2 mM PBS (pH 6.5) containing 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture and 0.1 M KCl from -0.5 to +0.7 V (vs. Ag/AgCl) at the scan rate of 50 mV/s to monitor the current variation (ΔI) before or after nanomaterial modification because Fc-MWCNT and Chi-PPy-AuNPs will increase the electron transferring and lead to current response enlarged (Gao et al., 2003). Moreover, the immunoreaction was monitored by amperometric measurements in PBS (pH 7.0) along with a small concentration of H_2O_2 purchased from Sigma. When the antigen was bound with antibodies: capture antibody coated on the surface of the electrode and the second antibody (labeled with HRP) catalyzing the substrate to generating current response, then there was current response of the immunoreactions. All the electrochemical experiment was carried at the room temperature.

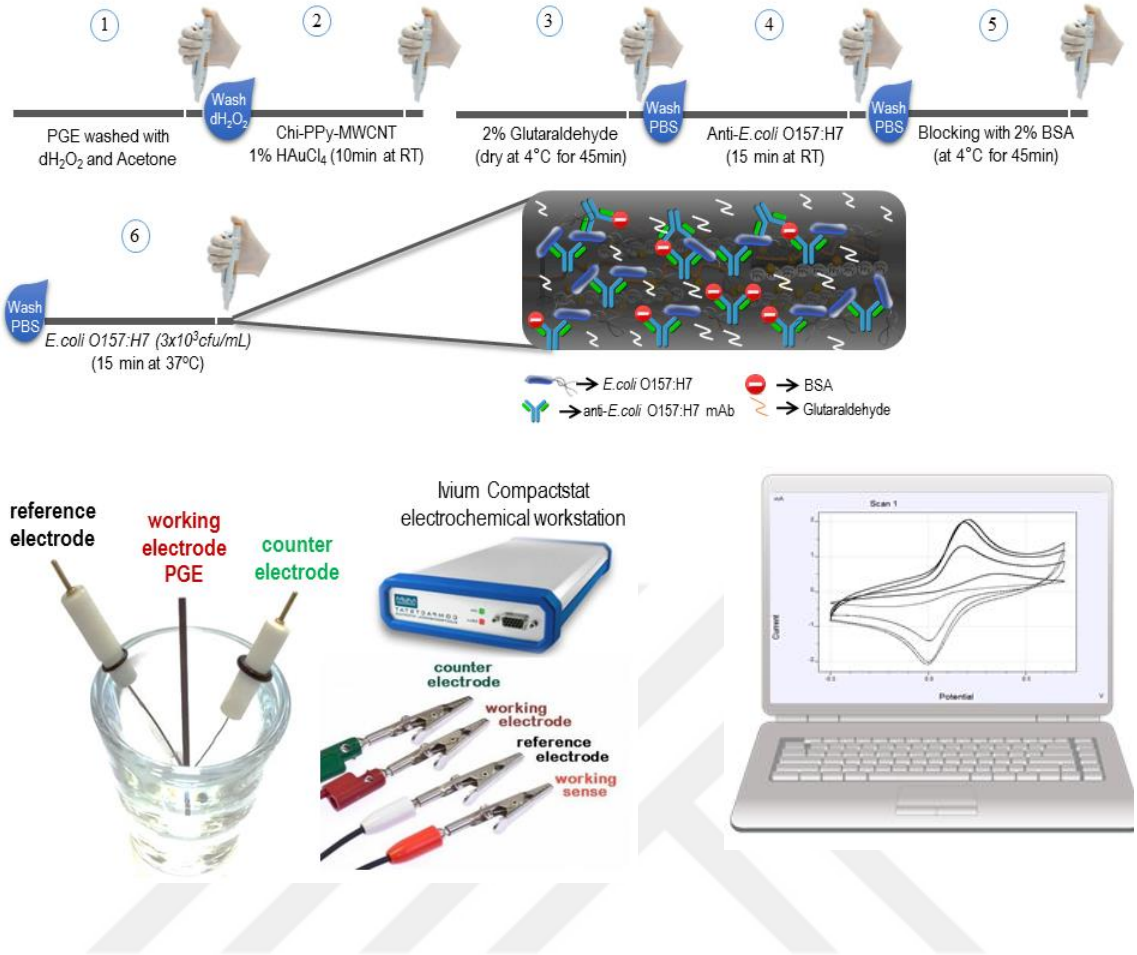


Figure 3.3 Schematic diagram of the experimental set up of an immunosensor fabrication.

3.9 *SANDWICH ELISA METHOD FOR E. coli O157:H7 DETERMINATION*

To prove our study, sandwich ELISA method was used. Various concentrations of heat killed *E. coli O157* cell (100ºC, 15 min in water bath) were used as a standard reference of the antigen. First a checker board titration was done in order to find the optimum concentration of antibody.

In sandwich ELISA method (Figure 3.4);

- 96 well ELISA plates (Greiner, Austria) were coated with 100 µL monoclonal antibody against *E. coli O157* solution containing 0.1, 0.5, 1, 5, 10, 50 and 100 µg/mL diluted with PBS.

PBS Buffer: For a stock of 500 mM phosphate (PO_4^-) buffer, 34.8 g K_2HPO_4 was dissolved in 400 mL dH_2O and 13.6 g KH_2PO_4 was dissolved in 200 mL dH_2O. Then, KH_2PO_4 solution was added into K_2HPO_4 slowly until the pH became 7.2 and was stored at 4ºC. 20 mL stock buffer was mixed with 980 mL dH_2O and 8.77 g NaCl.

- Plates were incubated for overnight at 4°C.
- Plates were washed 3 times with PBS-T by using EL_X50 Auto Strip Washer (BioTek, United Kingdom).

PBS-T: PBS buffer containing 0.05% Tween 20: 0.5 mL Tween 20 (Merck, Germany) was added to 1 L PBS buffer.

- Plates were coated with 200 µL, BSA (Fluka, Switzerland) in PBS for blocking non-specific sites incubated for 2 h at room temperature (RT).
- Plates were washed 3 times with PBS-T.
- 100 µL containing various numbers of *E. coli O157* cells (10^1 to 10^8 CFU/mL) with blocking solution was added and incubated for 30 min at RT.
- Plates were washed with 3 times PBS-T.
- Wells were incubated for 30 min at RT with HRP-conjugated polyclonal antibody (10µg/mL) in blocking solution (Sigma Aldrich, USA)
- Plates were washed 6 times with PBS-T.
- After the washing step, 100 µL TMB substrate buffer (Sigma Aldrich, USA) was added and incubated for 30 min in the dark at RT. The enzymatic reaction was stopped with 100 µL 1M HCl and the OD value was measured at 450 nm by Powerwave XS ELISA Reader (BioTek, United Kingdom) after 15 min following the addition of substrate.

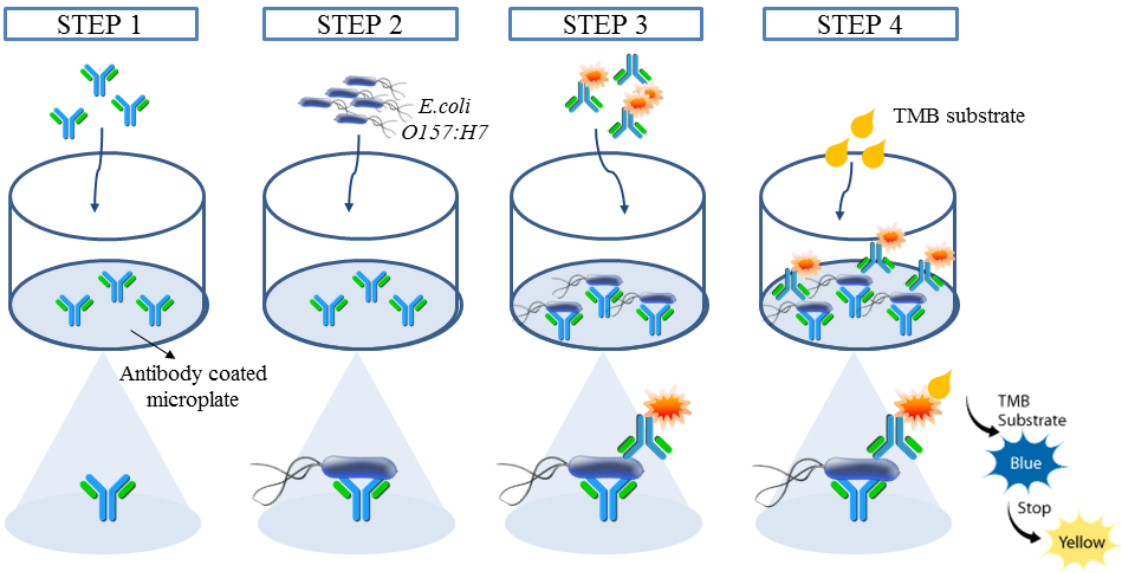


Figure 3.4 Schematic representation of sandwich ELISA procedure for the detection of *E. coli O157:H7.*

According to the results obtained by checker board titration, the concentration of the anti-*E. coli O157* monoclonal antibody to be used to coat plates was determined and similarly the fixed dilution of bacteria numbers to be used was determined.

3.10 *REAGENT OPTIMIZATION IN SANDWICH ELISA*

The concentration of the immune-reagents used in the ELISA method was optimized in order to achieve a good range of *E. coli O157* calibration curve that is so important for the determination of *E. coli O157* in the sample. The mAb for the coating layer, blocking reagent and HRP-conjugated antibody was optimized to accomplish the ideal concentration for the ELISA method. The concentration of mAb for the coating layer (0.1 µg, 0.5 µg, 1 µg, 5 µg, 10 µg, 50 µg and 100 µg in 0.05 M PBS pH 7.2), BSA blocking reagent (3%, 2%, 1% w/v in 0.05 M PBS, pH 7.2) and HRP conjugated pAb (0.1, 0.5, 1, 5, 10, 50 and 100 µg/mL) was optimized.

3.11 *SELECTIVITY AND CROSS REACTIVITY STUDIES*

Gram negative *Escherichia coli O124:NM* (ATCC 43893), *Pseudomonas aeruginosa* (ATCC 27853), *Shigella sp.* (ATCC 11126), *Burkholderia cepacia* (ATCC 25416) and *Salmonella enteritidis* (ATCC 4931) reference strains were used to determine the specificity of the optimized ELISA. A 100 µL of 5 µg/mL mAb against *E. coli O157* was immobilized on solid phase ELISA microtiter plate following the procedure described before for sandwich ELISA format. A 100 µL of each bacterial solution (10^6 CFU/mL) was used as the sample and incubated 2 hours at 37ºC. HRP conjugated polyclonal anti-*E. coli O157* (10 µg/mL) in PBS with 2% BSA was used as the detection antibody.

3.12 *REPEATABILITY AND STABILITY STUDIES*

The repeatability of the immunosensor was evaluated by calculating the relative standard deviation (RSD) value (standard deviation/mean x 100). Due to the fact that the precision of the immunosensor is generally evaluated regarding the statistical aspects such as variance, standard deviation and percent relative standard deviation (Epstein et

al., 2002). %RSD is commonly used in terms of easy calculation. The RSD of the analytical technique was conducted by performing 10 successive assays under the same conditions (explained in part 3.7) such as 10 µg/mL of mAb, 3 x 10^1 CFU/mL of bacteria solution and 5 µg/mL of HRP conjugated pAb. This immunoassay system was run by using the modified *PPy/AuNP/Fc/MWCNT@Chi* electrodes.

3.13 DETECTION OF *E. COLI O157:H7* IN REAL SAMPLES

Milk was purchased from a local supermarket. Sterilized milk was divided into 7 tubes and 100 µL of milk sample was filled into each tubes under a biosafety hood. Then, cultured *E. coli O157* with different concentrations from 3 x 10^1 to 3 x 10^7 CFU/mL were inoculated into sterilized milk. However, for eliminating lipids and proteins from the milk, proteinase K (Roche, Germany) and 50 µL of 0.1% Triton X-100 were added to 100 µL of milk, and the milk samples were incubated at 37^{o}C for 30 min to remove protein and lipid components. After this incubation, 900 µL of 150 mM NaCl was added and the tubes were shaken to mix the contents. Samples were centrifuged at 12,000 x g for 10 min and the bacteria-containing pellets were collected. The pellets were suspended in 1 mL of 150 mM NaCl and centrifuged again at 12,000 x g; the pellets were resuspended in 150 mM NaCl and used for detection of bacteria. All these procedures were summarized in Fig 3.5.

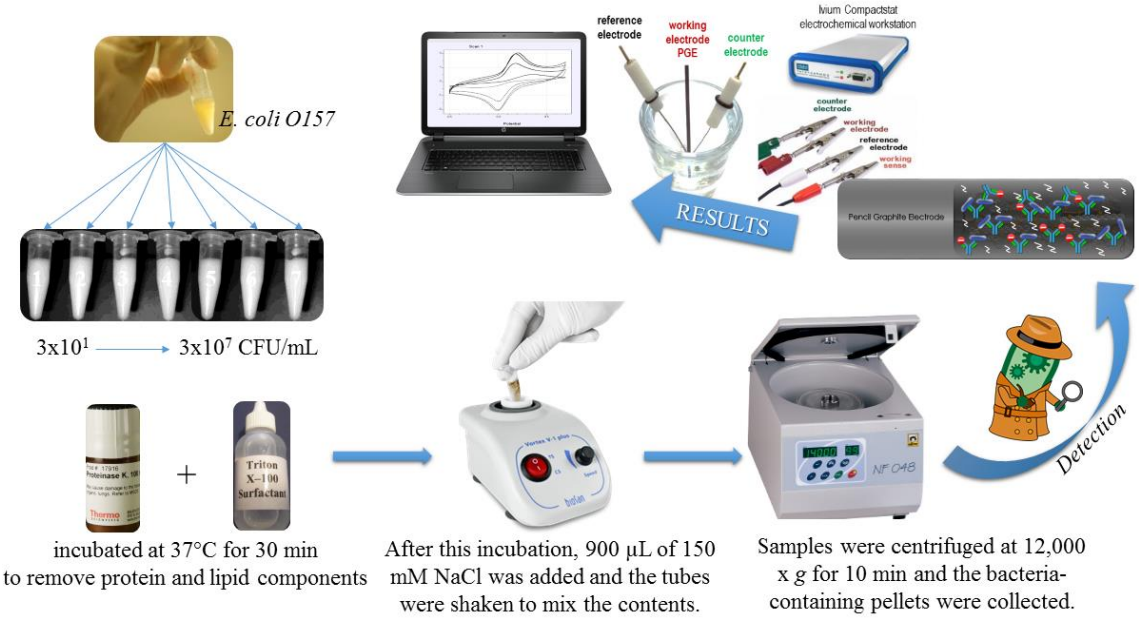


Figure 3.5 Schematic representation for the detection of *E. coli O157:H7* from the milk sample.

3.14 *SAFETY CONSIDERATIONS*

The target bacteria were inoculated the tryptic soy agar petri dishes and incubated at 37ºC for 20 h. The number of bacteria was enumerated using the conventional surface plate count method, and the cultures were then heated in a 100ºC water bath for 15 min to kill all the bacteria. The pure cultures were serially diluted to the desired concentrations with PBS for later use.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 *STANDARD CURVE OF ABSORBANCE AGAINST CFU/mL OF E. coli O157:H7*

In order to determine the number of *E. coli O157* cells in various serial dilutions, spread plate technique was used. This is a well-known technique for enumerating the bacteria which are able to grow on the agar medium and under the culturing conditions. After the incubation period, the number of colonies in the plate (typically between 30-300 colonies) is counted. Fig 4.1 represents that the colony formation of *E. coli O157* on the TSA medium with a dilution factor was 6. According to this result, he number of bacteria was determined by multiplying the number of colonies by the dilution factor.

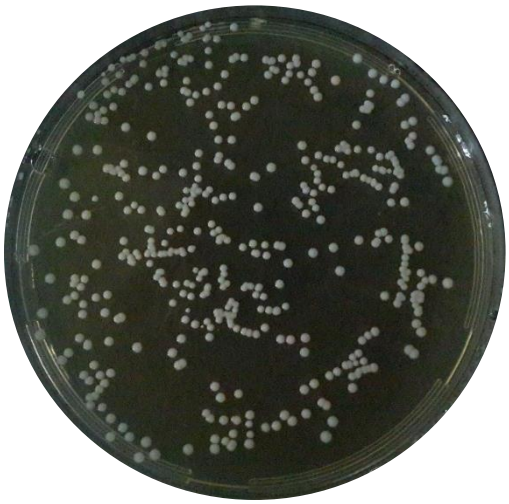


Figure 4.1 The typical appearance of *E. coli O157:H7* colonies on a tryptic soy agar plate. Colonies are small (0.5-2 mm in diameter), flavescent and water droplet-like.

In order to determine the concentration of CFUs (Colony Forming Units) in the bacteria suspensions, a standard calibration plot of absorbance against CFU/mL was constructed. The cell density of the bacteria suspensions was proportional with the CFU concentration after 24 h incubation (Figure 4.2). Concentration of the bacteria at 10^1 - 10^9 CFUs m/L showed the saturated density of the cell when the plot shows constant absorbance (600 nm at spectrophotometer (Shimadzu, mini 1240 UV)).

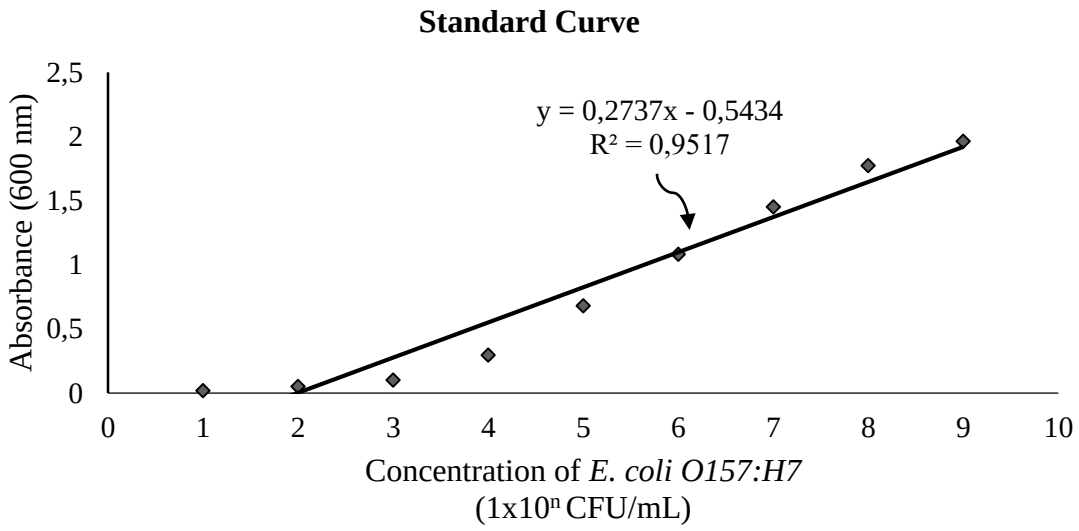


Figure 4.2 A standard calibration curve of *E. coli O157:H7* density at absorbance 600 nm against CFU/mL.

The standard curve graph was drawn according to the results of serially diluted *E. coli O157* solution and the linear trend-line was added to the data. R-squared value and the equations were displayed on the graph. The linear equation shown on the graph represents the relationship between Concentration (**x**) and Absorbance (**y**) for *E. coli O157*. The R-squared value (0.9517) is considered an acceptable estimation of the true relationship between concentration and absorbance. It is known that the closer to 1.0, the better the fit of the regression line.

4.2 *OPTIMIZATION OF THE CONCENTRATION OF THE ANTIBODIES IN SANDWICH ELISA METHOD*

In sandwich ELISA test, the microplate wells coated with 10 µg/mL of anti-*E. coli O157* monoclonal antibody and followed by blocking with 2% BSA in 0.05 M PBS pH 7.2 and then added different numbers of *E. coli O157* from 10^1 to 10^8 CFU/mL in PBS. anti-*E. coli O157* conjugated HRP polyclonal antibody at different concentrations 0.1 µg/mL, 0.5 µg/mL, 1 µg/mL, 5 µg/mL, 10 µg/mL, 50 µg/mL and 100 µg/mL in PBS were used before the addition of TMB substrate. Fig 4.10 illustrates the result of this test. Although the less number of bacteria and the low concentration of antibody, the OD values were achieved. Moreover, when increasing the number of bacteria and concentration of HRP conjugated antibody, it was clearly seen the absorbance could be read. 10 µg/mL anti-*E. coli O157* conjugated HRP polyclonal antibody was selected as an ideal concentration because of the peak value at a concentration of 1 x 10^4 CFU/mL of *E. coli O157*.

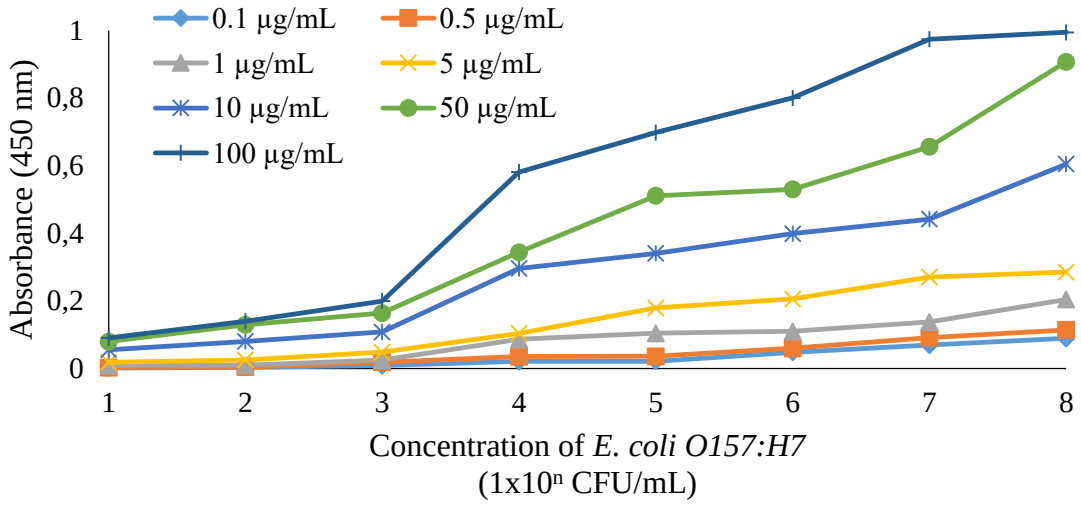


Figure 4.3 Sandwich ELISA technique to identify the optimal concentration of HRP conjugated antibody. Various concentration of anti-*E. coli O157:H7* conjugated with HRP

(0.1 to 100 µg/mL) was tested with fixed concentration of monoclonal antibody (10 µg/mL) and applied with *E. coli O157:H7* from 10^1 to 10^8 CFU/mL.

4.3 *DIFFERENT HEAT TREATMENT OF E. coli O157:H7 AND BINDING ABILITY WITH ANTI-E. coli O157:H7 ANTIBODY*

The binding ability of *E. coli O157* bacteria with its specific mAb after various heat treatments such as heat killed or without any treatment (live cells) was also investigated using sandwich ELISA technique. This experiment is to verify which structure of *E. coli O157* bacteria would generate a better signal and could be applied in the analysis of real food samples. Results showed that all the treatments had no effect on the binding ability to their antibody (Figure 4.4).

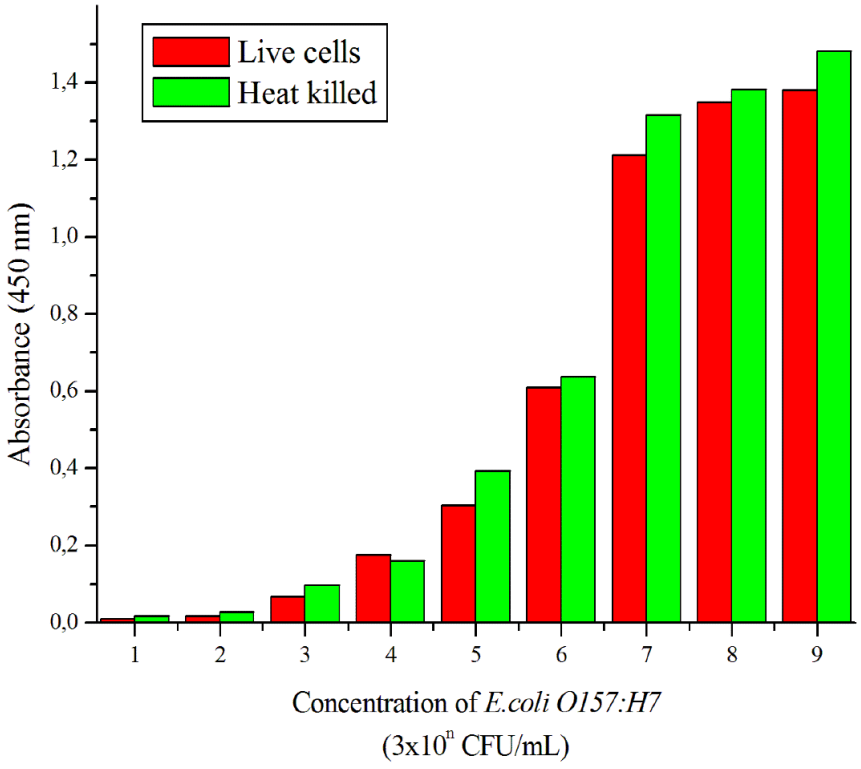


Figure 4.4 Evaluation of immunologic response of different treatment of *E. coli O157:H7* by ELISA method.

As it is understood from the Fig 4.4 that heat killed bacteria cells produced the good signal which was slightly higher than live ones. The results clearly showed that the contamination or infection was prevented in the laboratory during the study with these pathogenic bacteria. Therefore, this treatment was chosen for good safety precaution for further use.

4.4 *CYCLIC VOLTAMMETRY CHARACTERIZATION OF PPy/ANP/Fc/MWCNT@Chi MODIFIED ELECTRODES*

By using the CV for electrochemically characterizing the surface of the modified electrodes are so effective to apply in amperometric determinations. This characterization is based on the redox reaction of ferricyanide, as shown in equation below.

$$Fe(CN)_6^{4-} \longleftrightarrow Fe(CN)_6^{3-} + e^-$$

Fig. 4.5 shows the electrochemical characterization and optimization of differentially modified electrodes in 5mM $K_3[Fe(CN)_6]$ and 5mM $K_4[Fe(CN)_6$ at a scan rate of 50 mV/s. Among the well-formed redox peaks, it was clearly observed that the largest redox peaks (curve d and e) were belong to the *PPy/AuNP/Fc/MWCNT@Chi* electrodes. Curve b shows the bare electrode response of ferricyanide redox reaction. Curve a demonstrates the electrode after modified with only chitosan-polypyrrole composite and the redox peak slightly declined may due to inhibit the electron transfer. Curve c shows the Chi-PPy-Fc-MWCNT composite response and it increases the redox peak may due to its good conductivity properties. On the other side, curve d and e clearly shows the largest redox peak indication that the *PPy/AuNP/Fc/MWCNT@Chi* has the strongest ability of enhancing electron transfer among the others. This might be AuNPs increase the

conductivity of the film by facilitating the electron transferring. Because when increasing the amount of AuNPs, the response current was also increased as it is seen in curve e.

According to the CV results, modified PGE surface by *PPy/AuNP/Fc/MWCNT@Chi* could enable to transfer electrons for better electrochemical measurement and could denote the well-defined diffusion controlled redox wave characteristics.

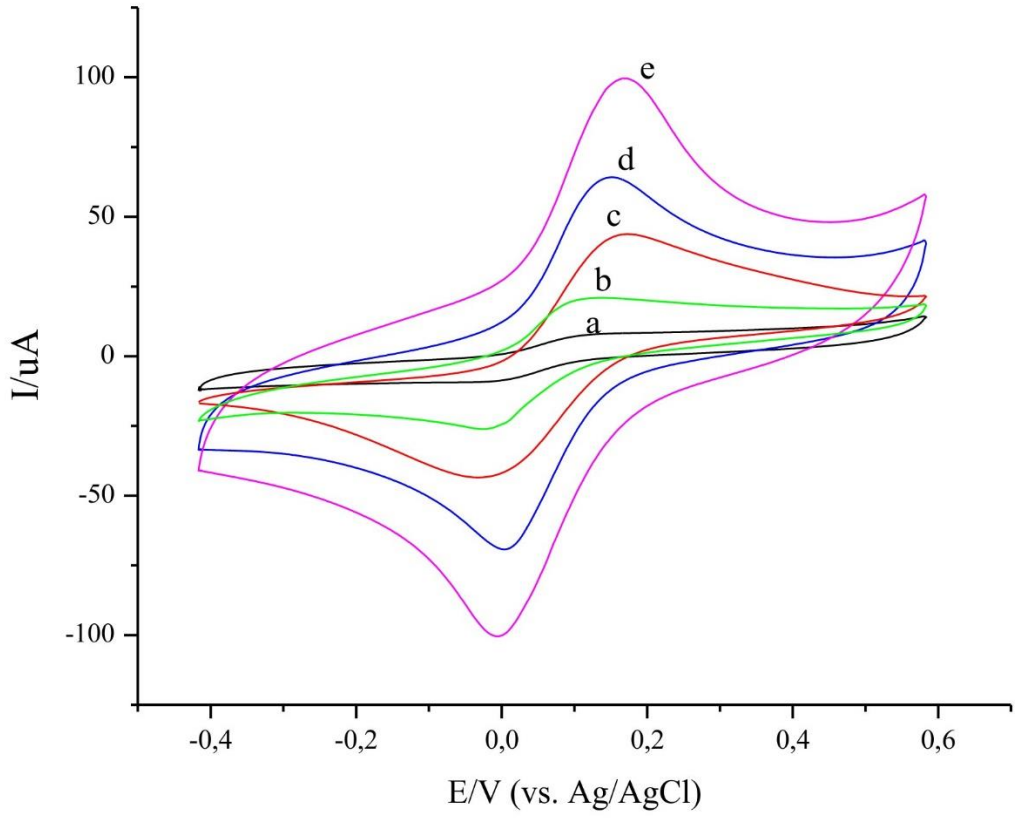


Figure 4.5 Cyclic voltammetry of different modified electrodes (a) Chitosan-PPy; (b) Bare electrode; (c) Chi-PPy-MWCNT; (d) Chi-PPy-MWCNT-AuNPs (1:1); (e) Chi-PPy-MWCNT-AuNPs (1:2) were obtained in 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ solution at a scan rate of 50mV/s.

4.5 *OPTIMIZATION OF THE CONCENTRATION OF ANTIBODIES IN AMPEROMETRIC ASSAY*

For enhancing the performance of the immunosensor, optimization of the capture antibody concentration has a great importance. Different concentrations of capture monoclonal antibody such as 0.1 µg, 0.5 µg, 1 µg, 5 µg, 10 µg, 50 µg and 100 µg/mL, specific to *E. coli O157* was studied on. Meanwhile, also the secondary polyclonal antibody with various concentrations like the monoclonal ones were used for this stage. When primary antibody concentrations less than 5µg/mL were used, an overall decrease in the final amperometric signal was observed due to insufficient bacteria capture. When concentrations more than 10 µg/mL were used, there were sufficient antibodies to capture the antigens and the final amperometric signal was high. However, a high antibody concentration increases the overall cost per assay. Optimum primary antibody was found to be 10 µg/mL therefore, a concentration of monoclonal anti-*E. coli O157* antibody 10 µg/mL was considered as the best compromise between effective bacteria capture and overall assay cost.

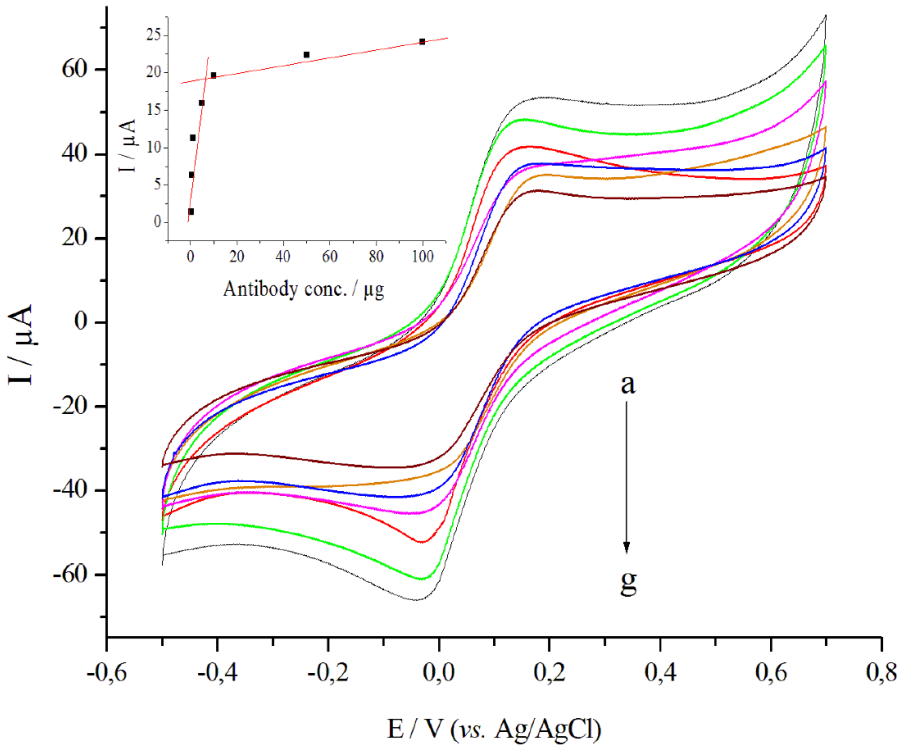


Figure 4.6 Cyclic voltammogram of the modified electrode with different concentrations of anti - *E. coli O157:H7* monoclonal antibody in 0.5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ from (a) to (g) represents different concentrations of antibodies (0.1 µg, 0.5 µg, 1 µg, 5 µg, 10 µg, 50 µg and 100 µg/mL, respectively).

Cyclic voltammetry studies of the different concentration of monoclonal anti-*E. coli O157* antibodies were performed between -0,4V - 0,6V at a scan rate of 50mV (Fig 4.6). As shown in the Fig 6, *PPy/AuNP/MWCNT@Chi* modified electrode shows the capture antibody concentration influence of the experiment results. In these cases, different concentrations of antibodies were immobilized onto the surface of the modified electrode. It is understood from the figure that when the concentration of antibodies coated on the surface of the modified electrode increased the current signals were decreased. That means antibody had been immobilized on the electrode surface and the reason for declining of amperometric current is that the transmission of electrons toward the electrode surface is

hindered by antibody. After that the blocking reagent BSA was then added to the electrode surface to prevent possible remaining active sites of the electrode and avoid the non-specific adsorption and binding. These concentrations were used in amperometric measurements and as a result 10 µg anti-*E. coli O157* showed good current response. For this reason, 10 µg anti-*E. coli O157* were used for capturing *E. coli O157.*

In order to detect *E. coli O157* at a concentration of 3 x 10^1 CFU/mL, various concentrations anti-*E. coli O157* polyclonal antibody conjugated-HRP were used and the amperometric signal was recorded. In this stage, the monoclonal antibody concentration was fixed as 10µg/mL during this optimization. When the concentration of HRP-conjugated polyclonal antibody was less than 5 µg/mL, the amperometric signal had been lost. This result suggested that the concentration of 1 µg/mL and lower were insufficient to get a signal during the measurement. Meanwhile, although different concentrations of *E. coli O157* were applied this optimization assay, there was no change in signal. On the other hand, when the concentration of polyclonal antibody was 5 µg/mL or higher, greater yield high magnitude amperometric signals were obtained. As a result, optimum HRP conjugated anti-*E. coli O157* polyclonal antibody concentrations were found to be 5 µg/mL providing a mediator between cost and sensitivity.

4.6 *DETECTION LIMITS OF E. coli O157:H7 WITH AMPEROMETRIC IMMUNOSENSOR*

In the CV analysis, the applied potential range was between −0.5 to 0.7 V with a scan rate 50 mV/s. 10 µL of bacterial solution including different numbers of *E. coli O157* in 0.1 M PBS pH 7.0 was dropped on the PGE surface. After 30 min incubation period, the excess of the bacteria was removed with PBS and HRP-conjugated anti-*E. coli O157* polyclonal antibody was added and incubated for 30 min. Then direct electrochemical signals of HRP at the resulting immunosensors were measured (Fig 4.7). The detection of *E. coli O157* was performed by measuring the change of reduction peak current response before and after the immunoreaction. The experiment was performed in PBS solution (pH

7.0) at 25 ± 0.5ºC. The decrease of the peak current for each sample was proportional to the concentration of pathogen in the incubation mixture.

Seven *E. coli O157* concentrations, from 3 x 10^1 to 3 x 10^7 CFU/mL were prepared by serial dilution in PBS buffer. A 10 µl sample of each bacterial suspension were taken for immunosensor to evaluate the detection range by calculation of ΔCurrent. The results which was depicted in Fig 4.7 that the concentration of bacteria and ΔCurrent were highly correlated. The linear relationship was well described (R^2 = 0.998) by ΔCurrent (µA) = 2.78 (log CFU/mL of *E. coli O157*)−1.16. The lower limit of detection was approximately 3 x 10^1 CFU/mL in PBS.

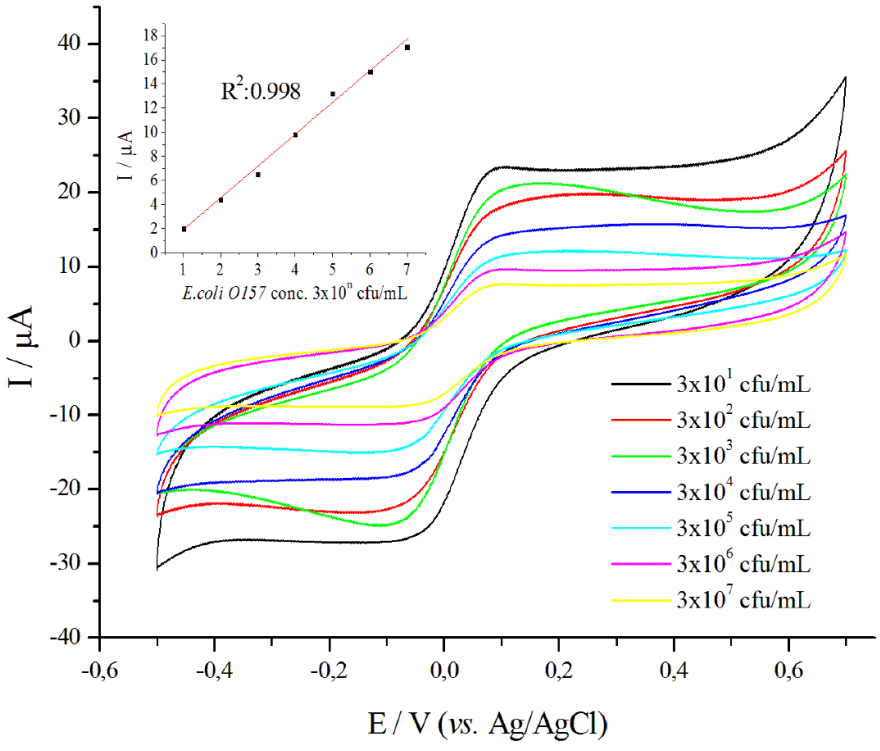


Figure 4.7 Cyclic voltammetry graphs of different *E. coli O157* concentrations and calibration graph obtained from oxidation peaks of modified electrodes due to the number of bacteria in 0.5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ solution at a scan rate of 50mV/s.

As in demonstrated in Fig 4.7, when anti-*E. coli O157* was immobilized to obtain anti-*E. coli O157 – PPy/AuNP/Fc/MWCNT@Chi* electrode, the current response was reduced, indicating that the antibody had been immobilized on the electrode surface. Immunosensor was incubated with different number of *E. coli O157* solution for 30 min, and the cyclic voltammogram exhibited an obvious decrease in the redox peak current. During the incubation process, the immobilized anti-*E. coli O157* reacted with *E. coli O157* in the solution to produce an immunocomplex that partially shielded the active center of the mediator and decreased its ability to convert electrons.

Moreover, we compare our results with the findings across the seven studies which were previously reported. As showed in Table 4.1, our results indicated that this method was more sensitive for the detection of *E. coli O157* as compared to the methods previously reported. Our linear range was $3 \times 10^1 – 3 \times 10^7$ and the limit of detection was 30 cfu/mL of *E. coli O157* effectively demonstrated the significant amplification *PPy/AuNP/MWCNT@Chi* electrode system.

Table 4.1 Performance compared with other references for the detection of *E. coli O157:H7.*

Detection Technique	**Linear Range (cfu/mL)**	**LOD (cfu/mL)**	**Reference**
Electrochemical immunosensor	$10^4 – 10^{10}$	4.570	Dou et al., 2013
Multiplex fiber optic biosensor	$10^1 – 10^9$	1.000	Ohk et al., 2013
Impedimetric immunosensor	$1.5 \times 10^2 – 1.5 \times 10^7$	150	Wang et al., 2013
Chemiluminescence immunoassay	$4.3 \times 10^3 - 4.3 \times 10^5$	1.200	Zhang et al., 2014
Electrochemical impedance spectroscopy	$10^3 – 10^7$	1.000	Li et al., 2014
Amperometric immunosensor	$3.6 \times 10^3 – 3.6 \times 10^6$	430	Cheng et al., 2014
Amperometric immunosensor	$3 \times 10^1 – 3 \times 10^7$	30	Present work

4.7 *HYDROGEN PEROXIDE RESPONSE CHARACTERISTICS OF THE IMMUNOSENSOR*

In order to demonstrate the relationship between the electrocatalytic reduction current and the concentration of H_2O_2, the amperometric measurements were performed. As it is known that H_2O_2 is the critical and necessary oxidizing element in HRP catalysis sensor.

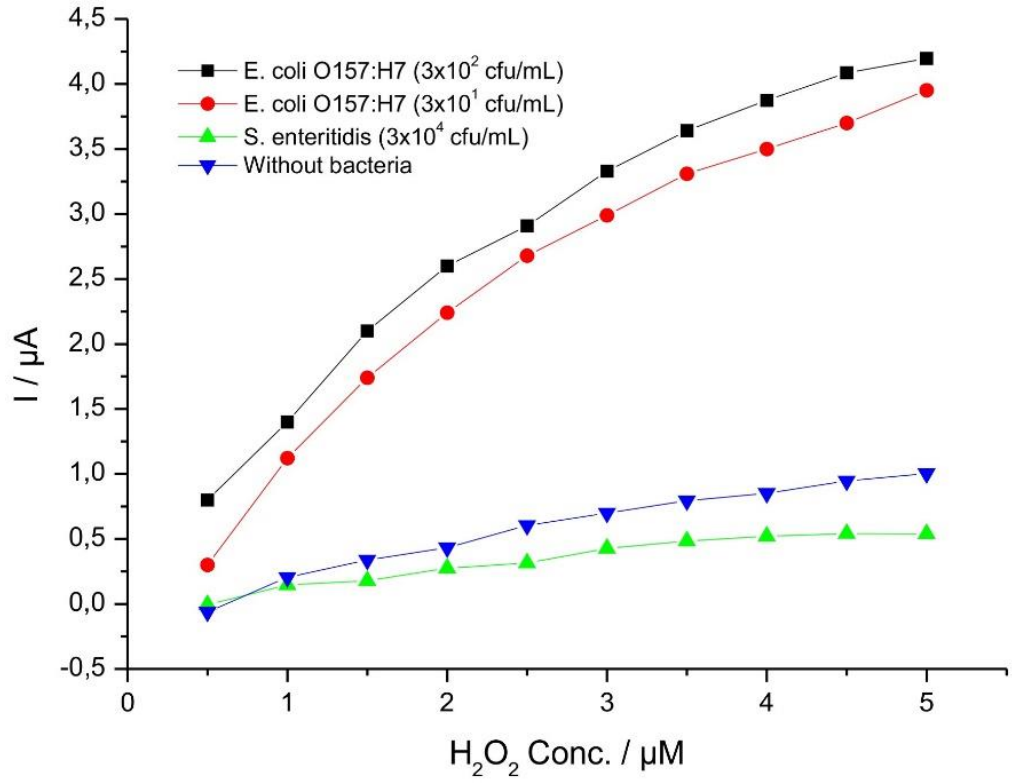


Figure 4.8 Calibration curves obtained from amperometric response of the *PPy/AuNP/MWCNT@Chi* electrodes at -0.3 V (*vs*. Ag/AgCl) upon successive H_2O_2 additions to different bacteria loaded electrodes.

In the CV analysis, the applied potential range was between −0.5 to 0.7 V with a scan rate 50 mV/s. 10 µL of bacterial solution including different numbers of *E. coli O157* in 0.1 M PBS pH 7.0 was dropped on the PGE surface. After 30 min incubation period, the excess of the bacteria was removed with PBS and HRP-conjugated anti-*E. coli O157* polyclonal antibody was added and incubated for 30 min. Then direct electrochemical signals of HRP at the resulting immunosensors were measured (Fig 4.8). The detection of *E. coli O157* was performed by measuring the change of reduction peak current response before and after the immunoreaction. The experiment was performed in PBS solution (pH 7.0) at 25 ± 0.5ºC. The decrease of the peak current for each sample was proportional to the concentration of pathogen in the incubation mixture.

The amperometry technique was performed in order to further illustrate the relationship between the electrocatalytic reduction current and the concertation of H_2O_2. The steady-state responses of the biosensor toward H_2O_2 were studied, and the linear response of the biosensor to H_2O_2 concentration ranged from 10 µM to 0.5 mM. As shown in Fig 8, there was no amperometric response of the *PPy/AuNP/MWCNT@Chi* electrode when there were no antigens during the injections of different H_2O_2 concentration at every in 25-30s intervals. However, when the presence of *E. coli O157* on the electrode, addition of H_2O_2 gave responses in PBS (pH 7.0). The results obtained in the presence of *E. coli O157* were compared to different pathogen such as *S. enteritidis.* Obviously, the results show that even the number of *S. enteritidis* higher than *E. coli O157*, there was no amperometric response obtained. Moreover, our *PPy/AuNP/MWCNT@Chi* modified electrode showed wider dynamic detection ranges corresponding lower detection limit.

4.8 *REPRODUCIBILITY, STORAGE AND STABILITY OF THE IMMUNOSENSOR STUDIES*

The reproducibility of the immunosensor was checked using the following method. A total of 10 different immunosensors were incubated under the same conditions and the same concentration of the *E. coli O157* (3 x 10^1 CFU/mL) solution.

A RSD value of 8.9% (Fig 4.9 A) was obtained for the measured reduction currents which indicating a good repeatability of such measurements with *PPy/AuNP/Fc/MWCNT@Chi* immunosensor. This demonstrated that the fabrication procedure for immunosensor designs was reliable and allowing reproducible amperometric responses.

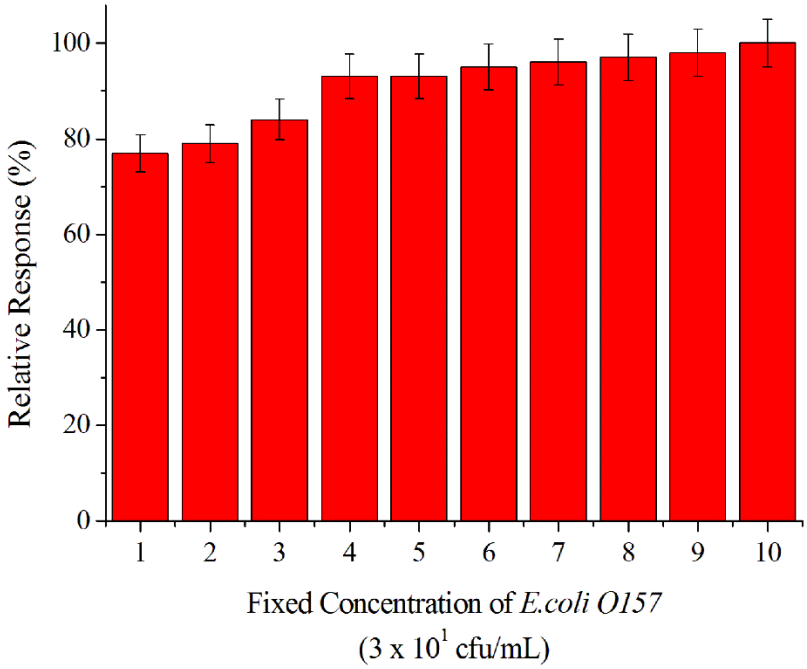


Figure 4.9 Measurements with fixed number of *E. coli O157:H7* (3 x 10^1 CFU/mL) with different electrodes. Error bar = standard deviation ,1.2, (n=10).

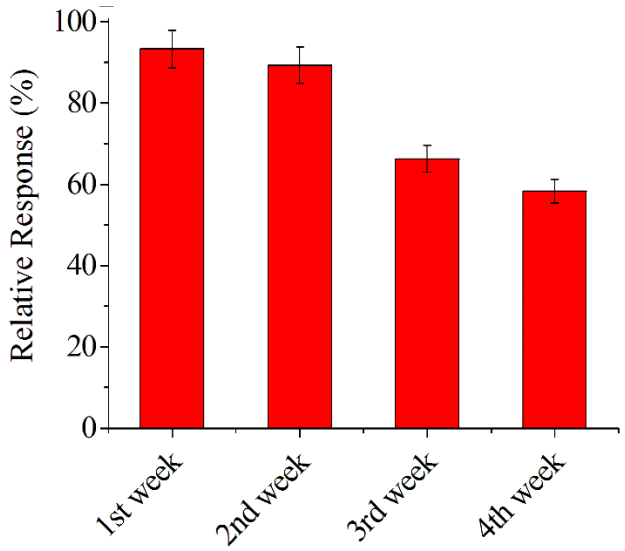


Figure 4.10 Stability of immunosensor for a period of 4 weeks at 4°C. The immunosensor response on a modified electrode by Amperometry at potential -0.3 V (*vs*. Ag/AgCl). Error bar = standard deviation, 2.5, (n=4).

For storage stability study, the electrodes were coated *PPy/AuNP/MWCNT@Chi* nanocomposites and monoclonal antibody and blocked with 2% BSA as described previously in the method section, stored at 4°C when it was not in use. The immunosensor was then used to detect the same concentration of *E. coli O157* at weekly intervals. The amperometric response of the immunosensor retained 93.3% (1st week), 89.3% (2nd week), 66.4% (3rd week) and 58.4% (4th week) of its initial sensitivity. The immunosensor possessed acceptable storage stability within the first 2 weeks (93.9% and 89.3%, respectively according to the initial response), however on the following weeks the current peaks dramatically declined (Fig 4.9 B). This might be due to the gradual deactivation of the immobilized biomolecules. Consequently, the results indicate that the immunosensor might be acceptable stability for two weeks. It should be noted that no stabilizing chemicals were used in these experiments.

4.9 *SELECTIVITY AND CROSS REACTIVITY OF THE IMMUNOSENSOR STUDIES*

To evaluate the specificity and reliability of the immunosensor, cross reactivity studies have great importance. Antibody plays an important role in cross reactivity due to it is specific to the antigen (Kawaguchi *et al.*, 2007, Salam and Tothill, 2009). Thus, the more specific a binding a site to the antigenic determinant, the more efficient the antibody-antigen reaction (Jongh-Leuvenink *et al.*, 2005).

In Fig 4.10 (A), the developed sandwich ELISA system showed high sensitivity for *E. coli O157:H7* (3 x 10^1 CFU/mL). The fabricated electrode immunosensor showed high sensitivity for *E. coli O157* (3 x 10^1 cfu/mL). The specificity of the assay was investigated in relation to other Gram negative bacteria with a fixed concentration of 3 x 10^7 cfu/mL such as *E. coli O124*, *P. aeruginosa*, *S. enteritidis*, *Shigella sp* and *B. cepacia* which are the most common bacterial contamination in food samples.

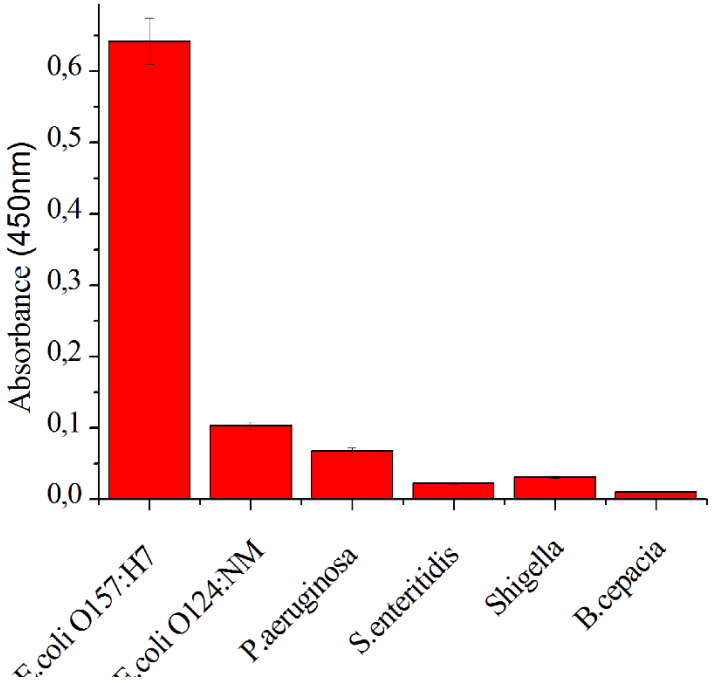


Figure 4.11 Specificity of *E. coli O157:H7* detection by the sandwich ELISA method. The cultured bacteria *E. coli O157:H7, E. coli O124:NM, P.aeruginosa, S. enteritidis; Shigella sp.* and *B. cepacia* at a fixed concentration (3 x 10^1 CFU/mL) were applied to the detection by sandwich ELISA method.

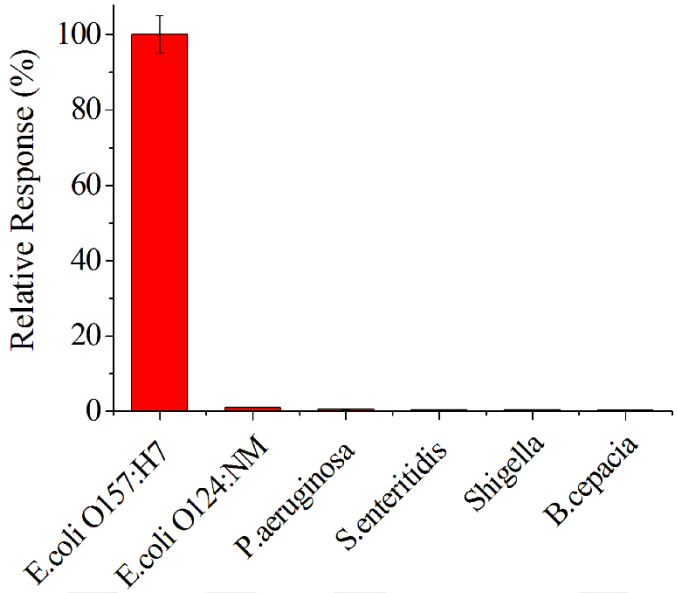


Figure 4.12 Selectivity of *E. coli O157:H7* detection by the *PPy/AuNP/Fc/MWCNT@Chi* electrode. Fixed number of *E. coli O157* (3 x 10^1 cfu/mL) were used in these immunosensing procedures. However, except *E. coli O157*, the rest of the other bacteria numbers were fixed as 3 x 10^7 cfu/mL. Each value was derived from three independent detections.

The selectivity of the proposed immunosensor was evaluated using the samples with bacteria *E. coli O157:H7, E. coli O124:NM, P.aeruginosa, S. enteritidis; Shigella sp.* and *B. cepacia* cultures at a concentration around 3 x 10^7 CFU/mL (Fig 4.10 (A)) in both sandwich ELISA method and immunosensing procedure. From the analysis of the results it can be concurred that our immunosensor is responsive to *E. coli O157* concentration of $3x10^1$ CFU/mL. That means it can successfully detect the presence of *E. coli O157* up to 3 bacterium/µL. The results were depicted in Fig 4.10 showed that *E. coli O157* was highly detected as compared to other bacteria. On the other hand, neither anti-*E. coli O157* mAb nor the pAb conjugated with HRP recognized the other bacteria such as *P. aeruginosa, S. enteritidis; Shigella sp.* and *B. cepacia.* In the case of *E. coli O157*, ΔCurrent reached 79.6

(±3.6) μA shown in Fig. 4.12 (B). However, the values of response current for the bacteria other than *E. coli O157:H7* were similar to the values obtained in the absence of bacteria, indicating that there was no obvious cross-reaction of *E. coli O157*-specific immunosensing electrode with other bacterial species or strains. According to overall results of ELISA and amperometric measurements visibly demonstrated that antibodies did not have nonspecific affinity for other Gram negative bacterial strains that were investigated.

Eventually, even at a high concentration (3×10^7 cfu/mL), the signal obtained was very low, thereby demonstrating the specificity of the system to different pathogens. Our fabricated immunosensor in this study demonstrated high sensitivity detection, good reproducibility, acceptable stability and high specificity.

4.10 DETECTION OF *E. COLI O157:H7* IN REAL SAMPLE STUDIES

The cultured *E. coli O157* cells were inoculated into milk at concentrations from $3x10^1$ to $3x10^7$ CFU/mL. These samples were used to detect *E. coli O157* by the *PPy/AuNP/Fc/MWCNT@Chi* electrode. A volume of 10 μL sample of each concentration was evaluated. The relative response and the concentrations of *E. coli O157* from $3x10^1$ to $3x10^7$ CFU/mL had a positive linear correlation shown in Fig 4.13.

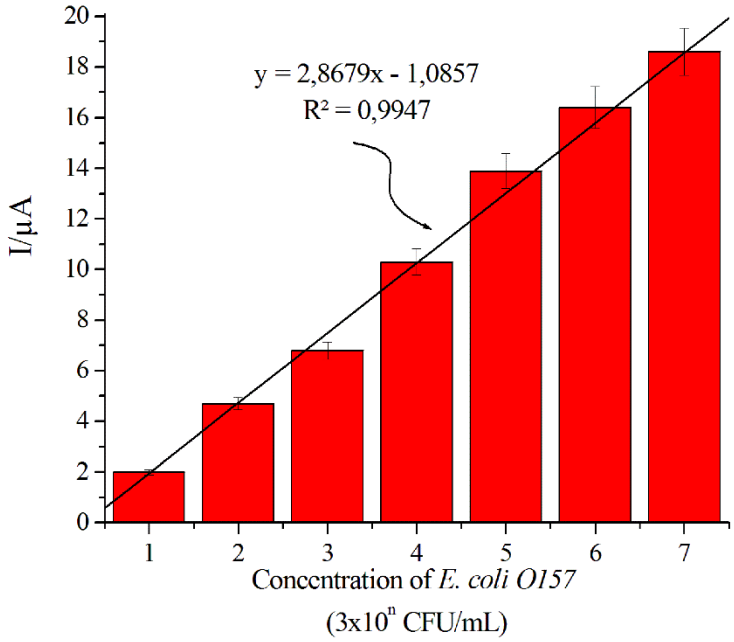


Figure 4.13 Detection of *E. coli O157:H7* in milk. Milk was inoculated with *E. coli O157:H7* cells at concentrations from $3x10^1$ to $3x10^7$ CFU/mL. The mean of each Δ Current (µA) was calculated from three independent measurements of PPy/AuNP/Fc/MWCNT@Chi electrode. In the detection by the immunosensor electrode, the values of Current and the concentrations of *E. coli O157* have a positive linear correlation (R^2=0,9947).

CHAPTER 5

CONCLUSION

In this electrochemical study, amperometric detection method was decided to select because of the several benefits such as ease of use, miniaturization and portable systems. In this work, a disposable nanocomposite film consisting of the *PPy/AuNP/MWCNT@Chi* has been designed to construct an electrochemical immunosensor for a sensitive immunoassay of *E. coli* O157:H7. The used our nanocomposite exhibits high current response intensity, good biocompatibility, and acceptable stability, which are demonstrated to be well competent for the development of electrochemical immunosensors. Electrode provides more accommodation for antibodies due to MWCNT that provides higher surface area on the electrode. In addition, for antibody immobilization, AuNPs are supported on the *MWCNT@Chi-PPy* composite films. In this case, it could improve the electrochemical signal and adsorption capacity of antibody and by this way enhance the detection sensitivity. Our developed immunosensor may provide a promising biosensor for detecting *E. coli* O157:H7 among the electrochemical biosensors in terms of the detection in food quality control, environmental monitoring and clinical analysis. The only difficulty is that the antibodies which are not able to be regenerated for subsequent detections thus it is not reusable.

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DECLARATION STATEMENT FOR THE ORIGINALITY OF THE THESIS

I hereby declare that this thesis comprises my original work. No material in this thesis has been previously published and written by another person, except where due reference is made in the text of the thesis. I further declare that this thesis contains no material which has been submitted for a degree or diploma or other qualifications at any other university.

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PUBLICATIONS AND PAPERS

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(2) Salih, B. A., Guner, A., Karademir, A., Uslu, M., Ovali, M. A., Yazici, D., ... & Arikan, S. (2014). Evaluation of the effect of cagPAI genes of Helicobacter pylori on AGS epithelial cell morphology and IL-8 secretion. *Antonie van Leeuwenhoek, 105*(1), 179-189.

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PROCEEDINGS

Salih BA, **Guner A**, Karademir A, Uslu M, Ovali MA, Yazici D, Bolek BK, Arikan S., "The cagPAI genes of *Helicobacter pylori* and their effect on the AGS cancer cell morphology and IL-8 secretion" International Symposium On Sustainable Development, Sarajevo, Bosnia and Herzegovina, May 15-18, 2014

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Ahmet GÜNER, "Developing an Electrochemical Immunosensor for the Detection of Food-Borne Pathogens Simultaneously", 2nd International Food R&D Brokerage Event, Swissotel Grand Efes, İzmir, Turkey, June 3-4, 2013

Bolek BK, **Guner A.** Arikan S, Salih BA, "Farklı Mide Patolojilerine Sahip Hastalardan İzole Edilen *Helicobacter pylori* cag PAI Çeşitliliğinin Mide Epitel Hücrelerine Etkisi.", 6. Moleküler ve Tanısal Mikrobiyoloji Kongresi, Ankara,Turkey, Jun. 15-19, 2010

A.Güner, G.A.Basli, G. Gyulai, Z.Tóth, Z.Szabó, L.Murenyetz, S.G.Yashina, V.L.Stakhov, L.Heszky, V.Gubin, "Light and Scanning Electron Microscopic Analysis of Silene stenophylla Seeds Excavated from Pleistocene-Age (Kolyma)", 18th International Electron Microscopy Congress ", Eskişehir, Turkey, Aug. 26-29, 2007

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