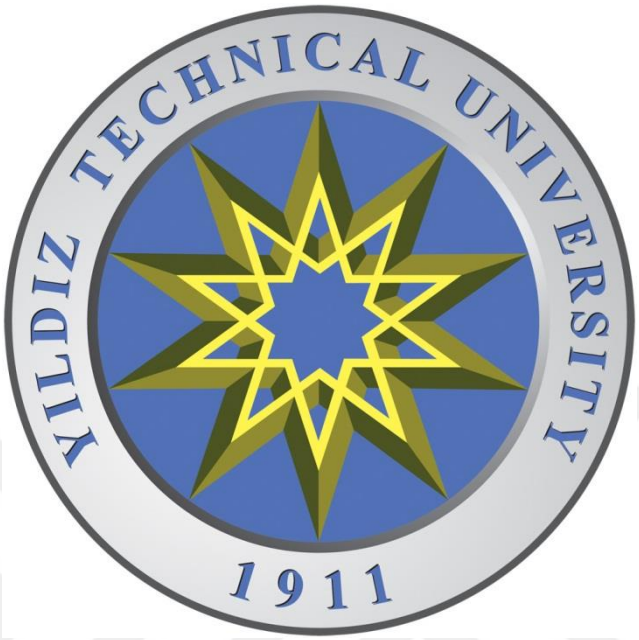

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

MSc. THESIS

DECEMBER, 2016

REPUBLIC OF TURKEY
YILDIZ TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DEVELOPMENT OF A LABEL-FREE ELECTROCHEMICAL IMPEDIMETRIC IMMUNOSENSOR BASED ON GOLD NANOPARTICLE MODIFIED SCREEN-PRINTED GOLD ELECTRODE FOR THE DETECTION OF CARCINOEMBRYOGENIC ANTIGEN (CEA) BY MONOCLONAL CEA ANTIBODY IMMOBILIZATION

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MSc. THESIS
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İSTANBUL, 2016

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A thesis submitted by Sultan ŞAHİN in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** is approved by the committee on 28.11.2016 in Department of Bioengineering.

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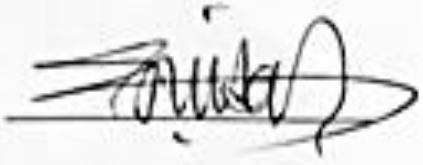

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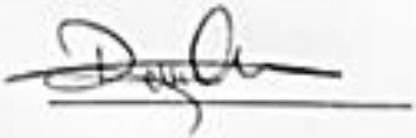

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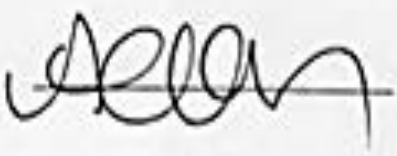

ACKNOWLEDGEMENTS

First, I would like to thank my parents, my brothers and my dear friends. All the support they have provided me over the years was the greatest gift anyone has ever given me.

Also, I would like to thank the Scientific and Technological Research Council of Turkey for its scholarship (TUBİTAK 2211-C) support.

December,2016

Sultan ŞAHİN

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LIST OF SYMBOLS

ΔE_p	Potantial difference
Δi	Current difference
$\Delta\varphi_m$	Difference potantial of metalic electrode
$\Delta\varphi_s$	Difference potantial of solution
$\Delta\varphi_{m/s}$	Difference potantial metalic electrode and solution
φ	Phase shift
ω	Angular frequency
σ	Warburg coefficient
C	Capacitance
C_{DL}	Double layer capacitance
$C_H^{1,2,3}$	Constant regions of heavy chain
C_L	Constant region of light chain
C_o	Concentration of O
D	Diffusion coefficient
D_o	Diffusion coeffiecient
E	Potantial
e^-	Electron
$E_{p,c}$	Cathodic peak potantial
$E_{p,a}$	Anodic peak potantial
F_{ab}	Fragment antigen binding
F_C	Fragment crystallisable
F_R	Fragment region
f	Frequency
$H_{1,2,3}$	CDR loops per variable domain on the heavy chain
H_C	Heavy chain
i_c	Capacitive current
i_f	Faradaic current
$i_{p,c}$	Cathodic peak current
$i_{p,a}$	Anodic peak current
k	Chemical reaction rate
$L_{1,2,3}$	CDR loops per variable domain on the light chain
L_C	Light chain
q	Charge
q_m	Charge density on metal electrode
q_s	Charge density of monomolecular layer in the solution
R_{CT}	Charge transfer resistance
R_{et}	Electron transfer resistance
R_p	Polarization resistance
R_{SOL}	Resistance of solution

t	Time
W	Warburg impedance
V_H	Variable region of heavy chain
V_L	Variable region of light chain
V_0	Amplitude of signal
V(t)	Applied potantial at t
v	Scanning rate
Y	Complex conductivity
Z	Impedance
Z_I	Imaginary part of impedance
Z_R	Reel part of impedance
Z_W	Warburg impedance

LIST OF ABBREVIATIONS

Ab	Antibody
AC	Alternating Current
Ag	Antigen
AgNPs	Silver Nanoparticles
Aq	Solution
ASV	Anodic Stripping Voltammetry
AuNPs	Gold Nanoparticles
BSA	Bovine Serum Albumin
CDRs	Complementary Determining Regions
CE	Counter electrode
CEA	Carcinoembriogenic Antigen
Chit	Chitosan
CNTs	Carbon Nanotubes
CPNWs	Conducting polymer nanowires
CV	Cyclic Voltametry
DC	Direct Current
DDC	N,N'-Dicyclohexylcarbodiimide
DMAP	4-Dimethylaminopyridin
DNA	Deoksiribonucleic acid
DPV	Differantial Pulse Voltametry
EIS	Electrochemical Impedance Spectroscopy
ELISA	Enzyme Linked Immunosorbent Assay
FBOS	Fiber optic biosensors
FET	Field Effect Transistor
FND	Ferrocenyl Naphthalene Diimide
GA	Gluteraldehide
GCE	Glassy Carbon Electrode
GHM	Gold Hollow Microshere
GOD	Glucose Oxidase
hCG	Human Chorionic Gonadotropin
HDT	Hexanedithiol
HRP	Horseradish Peroxidase
Ig	Immunoglobulin
IHP	Inner Helmholtz Plane
LOD	Limit of Detection
LSV	Linear Sweep Voltammetry
MWCNTs	Multiwalled Carbon Nanotubes
MDB	Meldola’s blue
MZI	Mach-Zehnder Interferometry

NSB	Nonspecific Binding
O	Oxidized
OECT	Organic Electrochemical Transistor
OFET	Organic Field Effect Transistor
OHP	Outher Helmholtz Plane
OTFT	Organic Thin Film Transistor
PAMAM	Poliaminoamine
PANI	Polyaniline
PBS	Phosphate Buffer Saline
PC	Polycarbonate
PSA	Prostate Specific Antigen
PtNPs	Platinium Nanoparticles
PVA	Polyvinyl Alcohol
QCM	Quartz Cristal Microbalance
R	Reduced
RE	Reference electrode
RIA	Radio immunoassay
RIgG	Rabbit Immunoglobulin G
SAMs	Self Assamble Monolayers
SAW	Surface Acustic Wave
SiNW	Silicon Nanowire
SPCE	Screen Printed Carbon Electrode
SPE	Screen Printed Electrode
SPGE	Screen Printed Gold Electrode
SPR	Surface Plasmon Resonance
SPW	Surface Plasmon Wave
TAA	Thiol aromatic aldehyde
WE	Working electrode

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ABSTRACT

DEVELOPMENT OF A LABEL-FREE ELECTROCHEMICAL IMPEDIMETRIC IMMUNOSENSOR BASED ON GOLD NANOPARTICLE MODIFIED SCREEN-PRINTED GOLD ELECTRODE FOR THE DETECTION OF CARCINOEMBRYOGENIC ANTIGEN (CEA) BY MONOCLONAL CEA ANTIBODY IMMOBILIZATION

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Impedimetric immunosensors are constructed with antibody immobilization of working electrode and their working principle is that occuring a correlation between antigen concentration and obtained resistance after an electrochemical antibody-antigen interaction. Electrochemical Impedance Spectroscopy (EIS) is generally used to characterize these type affinity detections in biosensor applications. Electrochemical impedimetric biosensors have significant advantages for sensitive detection of cancer biomarkers which are being smaller, faster, more sensitive, cheaper devices, without radiation hazards, allowing label-free, concurrent detection, simple production, less time consuming, rapid detection, having longer shelf life, and not complicated procedure. These properties will substantially get easier early diagnostic of cancer at beginning phases and examination of cancer disease in treatment progress. Carcinoembryogenic antigens (CEAs) which are cell surface glycoproteins are used as an important biomarker in human serum associated with colorectal, lung, breast cancer and ovarian carcinoma. CEA quantification analysis with electrochemical impedance spectroscopy promotes early diagnosis of cancer which is crucial for the successful treatment of the disease and increases health standards of people. The gold layer has various advantages during immobilization process thereby the easy adsorption of biomaterials relates to hydrophobic and thiol–gold interactions. In recent years, gold nanoparticles (AuNPs) are commonly used to enhance more sensitive electrochemical immunoassay for immobilization of antibody. AuNPs provide strongly adsorbtion of antibody on working

electrode during immobilization due to its large specific surface area, good biocompatibility, surface free energy of nanosized particles. AuNPs facilitate electron transfer between redox proteins and electrode surfaces, provide effective mass transport in electrochemical biosensor applications as making closer redox protein (monoclonal CEA antibody) to the electrode via nanosized structure. In the other words, AuNPs is a desirable intermediator for immobilization of antibodies. In this study, Screen-Printed Gold Electrode (SPGE) is modified with thiol and AuNPs to develop an impedimetric biosensor to detect CEA as an important cancer biomarker.

In this thesis, transducer part of the biosensor were studied because Au working electrode were being used as conductor in transducer part of biosensor. Modification of SPGE allowed an electrochemical change on transducer of our biosensor and this changes were converted to quantifiable signals by Electrochemical Impedance Spectroscopy (EIS) and Cyclic Votammogram(CV).

Key words: Electrochemical impedance spectroscopy, immunosensor, carcinoembryogenic antigen, tumor marker detection, antibody adsorbtion, gold nanoparticles

ÖZET

MONOKLONAL KARSİNOEMBRİYOJENİK ANTİJEN İMMOBİLİZASYONU İLE ALTIN NANOPARTİKÜL MODİFİYE EDİLMİŞ SERİGRAF BASKILI ELEKTROT TEMELLİ ETİKETLEME YAPMAKSIZIN İMPEDİMETRİK İMMUNOSENSOR GELİŞTİRİLMESİ

Sultan ŞAHİN

Biyomühendislik Programı

Yüksek Lisans Tezi

Tez Danışmanı: Prof. Dr. İbrahim IŞILDAK

İmpedimetrik immünosensörler, çalışma elektrodu üzerine antikor immobilizasyonu ile oluşturulurlar. Çalışma prensipleri ise birbirlerine spesifik antikor ve antijen çiftinin çalışma elektrodu üzerindeki elektrokimyasal etkileşiminden oluşan rezistans ile etkileşim gösteren antijen konsantrasyonu arasındaki ilişkiyi esas alır.Elektrokimyasal impedans spektroskopisi genellikle bu tip afinite biyosensör uygulamalarda kullanılmaktadır. Elektrokimyasal impedimetrik biyosensörlerin kanser biyomarkırlarının duyarlı tayininde önemli avantajları vardır; daha küçük, hızlı, duyarlı, ucuz ve radyasyonsuz olması, etiketleme yapmadan belirlenebilmesi, basit üretim prosedürlerine sahip olması, zaman tasarrufu sağlaması, daha uzun raf ömrünün olması ve kullanım basitliği bu avantajlardandır. Bu özellikler, insan kan serumundaki kanser markırı tespiti hastalığın erken aşamalarında tespit edilmesini ve takip aşamasındaki tetkikleri önemli ölçüde kolaylaştırır. Karsinoembriyojenik antijen (CEA), onkofetal bir antijendir, embriyonik dönemde oluşur ve doğumdan sonra baskılanır. Kan serumundaki miktarı kolorektal, akciğer, göğüs kanseri ve ovaryum karsinoma ile ilişkilendirilenönemli bir kanser biyomarkırıdır. Yetişkin insanların serumunda çok düşük düzeylerde bulunur. Yetişkin insanların serumunda yüksek düzeyde olması malignite bulgusu olarak kabul edilir. Elektrokimyasal impedans spektroskopisi ile CEA tayini hastalığın tespitini ve takibini kolaylaştırıp insanların yaşam standartlarını yükseltebilir.

Altın yüzey tiyol-altın ve hidrofobik etkileşimlerle alakalı olarak biyomateryallerin adsorbsiyonunu kolaylaştırma noktasında immobilizasyon sürecinde çeşitli avantajları

vardır. Son yıllarda, altın nanopartiküller antikor immobilizasyonunda, daha duyarlı bir elektrokimyasal immuno ölçüm yapılabilmesi için kullanılmaktadır. Altın nonopartiküller geniş yüzey alan oluşturabilme, biyouyumluluğu yüksek olması ile, nanoboyuttaki parçacıkların serbest yüzey enerjisine sahip olmasından dolayı çalışan elektrot üzerinde antikorların kuvvetli adsorbsiyonuna sebep olur.Altın nanopartiküller, elektrot yüzeyi ve redox proteini arasındaki elektron transferini hızlandırır, nanoboyuttaki yapısıyla redox proteinin (monoklonal CEA antikorunun) elektroda daha yakın olmasını sağlayıp elektrokimyasal biyosensör uygulamalarda daha etkili kütle transferi sağlar. Başka bir deyişle, altın nanopartiküller antikor immobilizasyonunda istenen bir aramoleküldür. Bu çalışmada altın çalışma elektrodu tiyolle modifiye edilip yüzeyi altın nanopartiküllerle fonsiyonelleştirilip CEA tespit edebilecek impedimetrik biyosensör geliştirmek hedeflenmektedir.

Bu tez çalışmasında biyosensörün transdüser kısmı üzerinde, Au çalışma elektrodunun elektrokimyasal etkileşimleri iletmesi temelinde çalışılmıştır.Serigraf baskılı altın elektrot modifikasyonu yüzeyde oluşacak elektrokimyasal değişimleri elektrokimyasal impedans spektroskopisi (EIS) ve dönüşümlü voltamogram (CV) ile değerlendirilebilir elektrokimyasal sinyallere dönüştürülmüştür.

Anahtar Kelimeler: Elektrokimyasal impedans spektroskopisi, immunosensor, karsinoembriyojenik antijen, tümör markır tespiti, antikor adsorpsiyonu, altın nanopartiküller

CHAPTER 1

INTRODUCTION

1.1 Literature Review

Biosensors are devices measure quantification changes of analyte which is intended detection. These measurement is provided a correlation between anayte concentration and transduction signal on transducer part of biosensor. Biosensors are mainly constructed three parts which are biomolecule/reseptor, transducer and detector/electronic part. Biomolecule/reseptor part recognizes analyte which is intended to detect in sample such as; body fluids, waste water or artificial mediums. Transducer part of a biosensor converts physical or chemical changes after interaction between biomolecule and analyte on transducer surface. Lastly, detector/electronic part of a biosensor displays the results as a quantifiable signal. Biosensors classification is based on immobilized biomolecule type on transducer surface and transducer type. Bioelement type based biosensors are enzymatic biosensors, immunosensors, nucleic acid based biosensors, microbial biosensors and carbohydrate biosensors. Transducer type based biosensors are electrochemical, optical, acustic, thermal and microcantilever biosensors[1].

IUPAC defines “electrochemical biosensor” following as; “Electrochemical biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element”[2]. Electrochemical biosensors are classified as potantiometric, amperometric, voltametric, conductometric and impedimetric biosensors. Electrochemical biosensors has an important place in the sensortechnology with various advantages which are having ease of fabrication and usage, adaptable for various analytes, portable, economical, fast and miniutarizable[3]. Impedimetric

biosensors among electrochemical biosensors are extensivety used in recent years due to their simple fabrication, more sensitive detection ability and portability. Impedimetric biosensors have also advantages to improve label free assesments. Label free assesments get easier detection procedure and get shorter response time of biosensors.

Electrochemistry has an important role in the research and development of biosensors. In biosensor application tree-electrode system is commonly used[4-9] with carbon pasta electrode, glassy carbon electrode, gold electrode and screen printed gold/carbon working electrode. Various modifications of working electrode have been studied in biosensor application for effective bioelement immobilization on working electrode[10], amplification of biosensor signals[11-14], increasing sensitivity of biosensors[15-18], decreasing response time of biosensors[14], [19].

Immunosensors is a type of biosensor classified according to sort of the immobilized bioelement on transducer. Bioelement used in immunosensors are immunological reagents (i.e. antibodies) which recognizes antigens. In the meantime, impedimetric electrochemical immunosensor developments have being in clinical applications, the idea of early and ultrasensitive screening of cancer biomarkers have promoted the enhancement of detection sensitivity and novel detection technologies by increased studies about ultrasensitive impedimetric cancer biomarker biosensors[20]. Impedimetric immunosensors is one of the most sensitive and selective in antigen quantification due to reaching very low detection limit by electrochemical impedance spectroscopy and high affinity between antigen-antibody molecules.

In fabrication an impedimetric immunosensor for cancer biomarker detection, working electrode modification is very significant key step to provide intended properties which are selectivity, sensitivity, low detection limit, economical, label free detection for point of care usage. Electrode modification is crucial step in fabrication of an immunosensor for antibody immobilization on working electrode of electrochemical biosensors. Various mateials as mediator, amplifier or linker have been used in the electrode modification. Due to highly conductivity, increasing surface area of substance, allowing effective immobilization, labellig Ab to amplification electrochemical signal, nanomaterials, conductive, semiconductive polymers or molecules have been favorable using in electrochemical immunosensor electrode modification.

Immobilization of bioelement on transducer surface of biosensor has a critical role in a biosensor fabrication progress. In biosensor applications, immobilization is to increase bioelement stability on transducer surface for increasing efficiency of physicochemical interaction between bioelement and analyte. Adsorption of bioelements on transducer surface is basic and easy method in the immobilization treatments. Adsorption is binding of molecules or particles on surface. Microencapsulation is another method in immobilization and it is also first used among others. In microencapsulation, bioelements are covered with a semipermeable membrane so that they are got more immobile on transducer surface. Entrapment is an immobilization method which provides to stabilize bioelements with gel matrix or polymer matrix. In this method, bioelements are stuck into the gel matrix or polymer matrix. Covalent binding of bioelements on transducer surface become with a covalent bond between functional group of bioelement and modified transducer surface with molecules could bind functional group of bioelement. This is also an immobilization method. Cross linking is another method which is provided in conjugation with microencapsulation method and adsorption method[1], [21].

Enhancements in immobilization techniques were observed with the nanotechnological developments. Researchers in their works implied that nanomaterials increase surface area of transducer surface which is covered with them and so that sensitivity of biosensor increase considerably[10], [22], [23]. By using nanomaterials and organic molecules in transducers of biosensors, we have an opportunity to their miniaturization and integration into portable electronic devices for medical applications of biosensors[24].

Nanoparticles have being commonly used for electrode surface modification taking advantages of the increasing surface area, biocompability, having good performance in mass transport or electron transfer between electrode and electrolyte solution. Metal, metal oxide, magnetic nanoparticles are commonly used in electrochemical biosensor applications. The gold nanoparticles among metallic nanoparticles are commonly used in electrochemical immunosensor working electrode modification to enhance surface area with other above mentioned advantages[6], [7], [25-28].

Gold materials are commonly used as biosensor transducer surface or in the transducer surface modification using gold nanoparticles with various shapes[29], gold nanorods and various composite with gold nanomaterials because gold materials have a number

of desirable properties which are essentially inert, non-toxic and biocompatible. Gold layer obtains easy adsorption of biomaterials relates to hydrophobic and thiol–gold interactions during immobilization process[30]. In recent years, gold nanoparticles (AuNPs) are commonly used to enhance more sensitive electrochemical immunoassay for immobilization of antibody. AuNPs provide strongly adsorbtion of antibody on working electrode during immobilization due to its large specific surface area, good biocompatibility, surface free energy of nanosized particles[31], [32]. AuNPs facilitate electron transfer between redox proteins and electrode surfaces, provide effective mass transport in electrochemical biosensor applications as making closer redox protein (Ab) to electrode via nanosized structure. In the other words, AuNPs is a desirable intermediator for immobilization of bioelements[33].Colloidal AuNPs solution as biolinker have been extensively prepared for immobilization of antibodies to costitude AuNPs layer on thiol modified working electrode[34].

Thiol layers are significants and extensively being used to modify metal surface asspecially gold surface because of extremely high affinity between gold and sulfur atoms. Adsorbtion of molecule with thiol can be constructed by Self Assamble Monolayer (SAMs) technique[35]. In recent years, there is mixed self-assembled monolayers because construction of mixed SAMs interfaces by co-adsorbing thiols of different chain length or end group functionality provide a controlled chemistry and structure. Mixed SAMs using alkanethiols of different chain length or end group functionality increase antibody immobilization efficiency by enhancing functional surface area and reducing reducing steric hindrance [36].

Modification of working electrode with collaidal AuNPs helps oriantation of proteins on gold nanoparticles conveniently. AuNPs morphology and distance between particles are important factors which at connection between working electrode and redox proteins. Blocking reagents are employed to block the possible remaining active sites of the nano-Au monolayer, avoid the non-specific adsorption of antibody in immobilization process and so they amplify the signal response of the antigen–antibody reaction on electrode. Commonly used blocking reagents on nano Au layer are BSA, HRP and ethanolamine[8], [37-39].

In process of the immunosensor fabrication, Ab immobilization has a critical point to obtain selectivity and sensitivity of biosensor. To bind Ab on electrode surface, there have to be surface modification by various mateials and maintance Ab binding on

electrode with right oriantation. If the oriantation of Ab on transducer surface can not allow the binding to Ag, desired immunosensor design could be unsuccesful beause of loss of biochemical interaction of Ab-Ag as increasing backgroundsignal. Complementary antigen binding sites of immobilized antibody have to be allow efficiently binding as well as to antigen and therefore Abs cannot be randomly oriented on the surface. It is named nonspecific binding(NSB) which involves the adsorption of conjugated enzyme or other labels used for immunoassay to materials other than the analyte[40].

Carcinoembryonic antigen (CEA) was discovered by Gold and Freedman in 1965 by isolation from adenocarcinoma. CEA molecule is in family of cell surface glycoproteins, a subfamily among Immunoglobulin Superfamily[41]and it is used as an important biomarker in human serum associated with colorectal, lung, breast cancer and ovarian carcinoma. Molecular weight of CEA is about 200 kDa[42]. CEA is composed by 641 aminoacids which are primarily included asparagin, glutamin, treonin and serin aminoacids. Major monosacharide part of the CEA molecule (2/3 of whole complex CEA molecule) is consist of N-acetil glucoseamine[43].

It is thought that CEA is metabolized in the liver however there isn't common view about the catabolizm of CEA molecule according to recent studies[44]. CEA is an oncofetal antigen which generates in the embryonic stage and is repressed in the postnatal stage. Mature human serum includes CEA at very low level. If there is high level of CEA in the mature human serum, it is recognized as malignite signs[45].

CEA molecule as a tumor marker is first defined in colon cancer and colorectal cancer specifically. However, it was determined that CEA level can be high in other cancer types and some pathological stuation out of cancer. In the cancer researches and treatments, CEA is the most frequently used tumor marker[44]. For instance, after the demonstration of CEA secration ability in the lung cancer process, it was investigated place of the CEA in lung cancer diagnosis, staging and prognosis estimation. In the process of non-small cell lung carcinoma, CEA is propounded as a benign tumor marker[44].

CEA can penetrate to body filuid due to being on cell surface. 97% of healthy and non-smoker people have less than 2,5 ng/ml CEA in serum. 19% of excessive smokers and 7% of former smokers have more than 5 ng/ml, and this is approved as a meaningful

data in the cancer phenomena. Higher level than 20 ng/ml of CEA in serum indicates metastasis stage of cancer disease and cancer prognosticate of some cancer types such colon and pancreas[46].

CEA quantification analysis with electrochemical impedance spectroscopy promotes early diagnosis of cancer which is crucial for the successful treatment of the disease and increases health standards of people.

1.2 Objective of the Thesis

In this study, Screen Printed Gold Electrode (SPGE) was thiolated with 1,6-hexanedithiol and modified with AuNPs to increase surface area of gold working electrode.After gold nanoparticle synthesis, morphological analysis was performed with FESEM. UV-vis spectroscopy was applied and Zeta potantial of colloidal gold solution gave information about gold nanoparticles sizes. Synthesised AuNPs by Frens method produces AuNPs with reducing gold ions in $HAuCl_4$ by citrate molecules. The reduced gold atoms in solution, aggregate and form clusters, these clusters grow by increasing the number of atoms until achive the particles form. In AuNPs synthesis, trisodium citrate acted as a capping agent and thus restricted the growth of Au[47]. UV-vis spectrum gave about 520 nm and FESEM images implied that sythesized colloidal gold nanoparticles are about 40-45 nm.

On the gold electrode surface, the self assable monolayer of chemisorbed 1,6 hexanedithiol has beed used as the base interface for the deposition of synthesised Au colloid by Frens method[48] onto Au surface. Thiol group of 1,6 hexanedithiol strongly binds gold surface due to the soft character of both Au and thiol[49]. Other thiol group of 1,6 hexanedithiol was free to bind with Au colloids introduced in solution. After surface modifications, monoclonal anti-CEAs were adsorbed on AuNPs deposited gold surface. Electrochemical characterization methods performed after each modification and anti-CEA – CEA interaction. These electrochemical characterization methods were cyclic voltametry and electrochemical impedance spectroscopy.

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CEA monoclonal antibodies were immobilized to detect carcinoembriogenic antigens. Antigens which are intended to detect are Carcinoembriogenic antigen (CEA) cancer biomarkers. Detection of CEA lung cancer biomarker from blood provides an early fast diagnose for lung, pancreas and colon cancer diseases. Because these cancer diseases are cancer types that more insidious spread in the human body, a fast and early diagnostic is very important for cancer treatment. In this thesis, by using electrochemical characterization methods; it was aimed that CEA detection was provided on AuNPs modified SPGEs. Obtained detection of 100 ng/ml, 50 ng/ml and 25 ng/ml showed that portable SPGEs could be modified to develop a label-free impedimetric immunosensor.

In conclusion, this study implies that CEA detection on modified SPGE can be performed without labelling any molecule. Label free assesments get easier detection procedure and get shorter response time of biosensors.This can be promoted as an label free impedimetric immunosensor for follow-up care of lung cancer. Using SPGE in development an immunosensor gets easier usage of the immunosensor as portable and in pont of care. Development of a label free impedimetric immunosensor based on Ab immobilization on SPGE can lead to commercilization of these types of portable biosensor developments for other protein detections.

1.3 Hyphothesis

Impedimetric immunosensors are constructed with antibody immobilization of working electrode and their working principle is that occuring a correlation between antigen concentration and obtained resistance after an electrochemical Ab-Ag interaction. EIS is generally used to characterize these type detections in biosensor applications.

Electrochemical impedimetric biosensors have significant advantages for sensitive detection of cancer biomarkers which are being smaller, faster, more sensitive, cheaper devices, without radiation hazards, allowing label-free, concurrent detection, simple production, less time consuming, rapid detection, having longer shelf life, and not complicated procedure. These properties will substantially get easier early dianostic of cancer at beginning phases. Carcinoembryogenic antigens which are cell surface glycoproteins are used as an important biomarker in human serum associated with colorectal, lung, breast cancer and ovarian carcinoma. CEA quantification analysis with electrochemical impedance spectroscopy promotes early diagnosis of cancer which is crucial for the successful treatment of the disease and increases health standards of people. The gold layer has various advantages during immobilization process thereby the easy adsorption of biomaterials relates to hydrophobic and thiol–gold interactions. In recent years, gold nanoparticles (AuNPs) are commonly used to enhance more sensitive electrochemical immunoassay for immobilization of antibody. AuNPs provide strongly adsorbtion of antibody on working electrode during immobilization due to its large specific surface area, good biocompatibility, surface free energy of nanosized particles. AuNPs facilitate electron transfer between redox proteins and electrode surfaces, provide effective mass transport in electrochemical biosensor applications as making closer redox protein (monoclonal CEA antibody) to the electrode via nanosized structure. In the other words, AuNPs is a desirable intermediator for immobilization of antibodies. In this study, the gold electrode is modified with thiol and AuNPs to develop an impedimetric biosensor to detect CEA as an important cancer biomarker.

1,6 hexanedithiol Self Assamble Monolayer on SPGE was constructed help of extremely high affinity between gold and sulfur atoms as a linker to AuNPs adsorbtion.AuNPs facilitate electron transfer between redox proteins and electrode surfaces, provide effective mass transport in electrochemical biosensor applications as making closer redox protein (CEA Antibody) to electrode via nanosized structure. Semicircle diameter of EIS Nyquist plots impliedcharge transfer resistance, Rct and this electrochemical change are correlated with detected monoclonal CEA antibody on AuNPs modified SPGE surface. In this study, it is implied that AuNP is a desirable intermediator for immobilization of CEA antibody as bioelement of immunosensor. Electrochemical impedimetric biosensors are more convenient to development of label free detection.

CHAPTER 2

THEORITICAL BASIC CONCEPTS

2.1 Electrochemistry

Electrochemistry is a science which incdudes reactions producing electric energy or occuring electron transfer by electric energy. These electrochemical reactions occur between electrolyte components in two ways those are loss of electrons (oxidation) and gain of electrons (reduction) when a sufficiently positive or negative potential is applied, respectively. In the case of electrochemical reactions, oxidation and reduction reactions proceed together and are called redox reactions. After redox reactions, some prominent electrochemical analyzer provide informationabout theanalyte concentration, reaction mechanisms, kinetics, and other behaviorof a chemical species in electrolyte solution by using some electroanalytical information about stoichiometry of charge-transfer at the substances' interfaces, mass transfer rate, adsorbtion, rate of chemical reaction and equilibrium constant of reaction.These dynamics of electrochemical reactions are effected by electrode surface and constituent, reactivity of species in electrolyte. Changing charge transfer process rate by all these factors effects redox reaction dynamics. Therefore, these factors must be analyzed carefully. Electroanalytical analyze methods have advantages such as; being cheaper, high sensitivity, using easily, fast and accurate response, being spesific for an almost definite element or molecule oxidation step.

Electrochemical reactions occur in electrochemical cell whereby electrons transfer from one material to another in an approptiate system. Electrochemical reactions system comprises an buffer solution which includes interested analyte and provides electrical conductivity of electrodes on which electrochemical interactions occur, in two or three electrode system and transducer which combines electrodes and monitor to display electrochemical signal[50], [51].

In two-electrode system, there are working and nonpolarized reference electrodes; in three-electrode system, there is additionally an axuiliary electrode (Figure 2.1). Electrodes are immersed in buffer solution containing the analyte and a supporting electrolyte solution in electrochemical cell. An electrochemical cell potantial is determined by difference between cathode and anode potantials. In tree-electrode system, potantial of electrochemical cell is determined by potantial difference of working electrode and reference electrode. Working electrodes are microelectrodes on which analyte is oxidized or reduced at linearly changing potantial with time. Working electrodes are usually constructed from inert materials such as; carbon, graphite, glassy carbon, platinum, gold, mercury, bismuth, palladium. As independent from analyte solution components, reference electrodes provide a fixed potantial against applied potential to the working electrode and they consist of saturated calomel electrode, Ag/AgCl electrode, standard hydrogen electrode or normal hydrogen electrode and Hg-Hg_2SO_4 electrode. The auxiliary electrode is also refered as counter electrode and serves completion of the current path. It generally consists of a platinum wire. The three electrodes are connected to a potentiostat/galvonastat device, which is transducer and controls the potential applied to the working electrode, and the results are monitored and recorded by a computer[51].

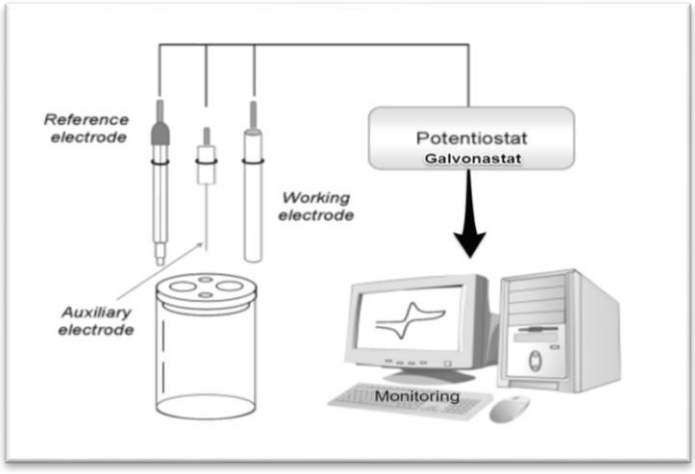

Figure 2.1Schematic of basic tree-electrode system [52]

Screen printed electrodes(SPE) get in favour over the past decade due to high electrocatalitic activity, perfect compability, high sensitivity, stability, easy of use and low cost properties. SPE has provided ease of use fairly whereby working, reference and auxiliary electrodes can be printed on the same substrate surface (Figure 2.2). This

fabrication design also allows to develop portable and rapid electrochemical biochips for point of care[53].

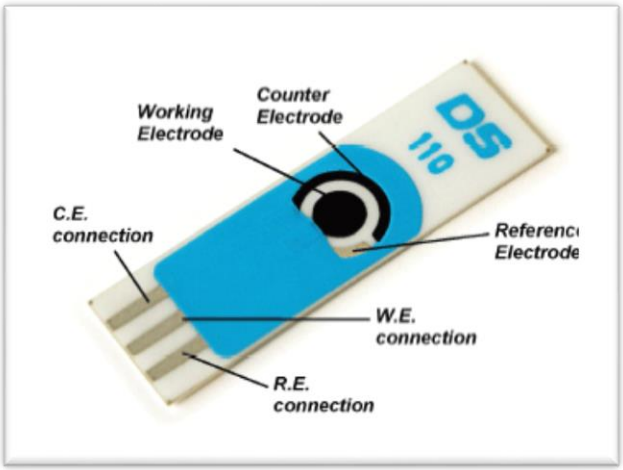

Figure 2.2 Screen Printed Electrode (DropSens)

In the case of an electrochemical reaction with metalic electrode and electrolyte solution which includes reactant molecules, a charge transfer occurs in the electrode/solution interface. This reaction has reached equilibrium after some time and charge transfer creates a potantial difference between two phases (Equation 2.1). Potantial difference between metalic electrode and electrolyte solution is showed in equation 2.2, where *aq* is solution, $\Delta\varphi_{m/s}$ is difference potantial metalic electrode and solution, $\Delta\varphi_m$ is potantial of metalic electrode, $\Delta\varphi_s$ is potantial of solution at the interface. With applying polarization potantial, change on electrode surface can be controllable and electrostatic interaction at the electrode/solution interface can be increased.

$$Fe^{3+}(aq) + e^{-} \rightarrow Fe^{2+}(aq) \quad (2.1)$$

$$\Delta\varphi_{m/s} = \Delta\varphi_m - \Delta\varphi_s \quad (2.2)$$

Helmholtz basicaly suggested a model to introduce electrical double layer in 1879. According to this model, overcharges on the metal electrode are neutralized by monolayer opposite charges in the solution. Occured neutral surface implies that charge density on metal electrode (q_m) is equal charge density of monomolecular layer in the solution (q_s). Other layer is comprised by counterions in the solutions and seperated by Outher Helmholtz Plane (OHP). Helmholtz theorized that a solvation shell could form between ions and electrode, and attracted ions by electrode can neighbour until boundered distance by the solvation shell in 1910. Thus, decrease of the potantial is

linear between OHP and electrode. In addition to Helmholtz model, Gouy and Chapman introduced Brownian motion of ions. If the ions can move without constraint, this ions are not proper for OHP. Thus, the ions have Brownian motion loses electrostatic interaction to electrode surface and henceforward, diffusion layer had been began and ions disperse in the diffusion layer. In 1924, Stern extended this model as combining Helmholtz and Gouy-Chapman model which is taken into considiration of ion molecules dimentions and that ions are covered with H_2O molecules. Therefore, ions can not attach to electrode surface and potantial dicrease is not as in Helmholtz and Gouy-Chapman model. Unsolved ions or ions without solvation shell can attach more than soluble ions. Thus, ions which are directly connected on electrode are specificaly adsorbed and Inner Helmholtz Plane (IHP) have been formed. On the other hand, soluble ions form OHP (Figure 3).

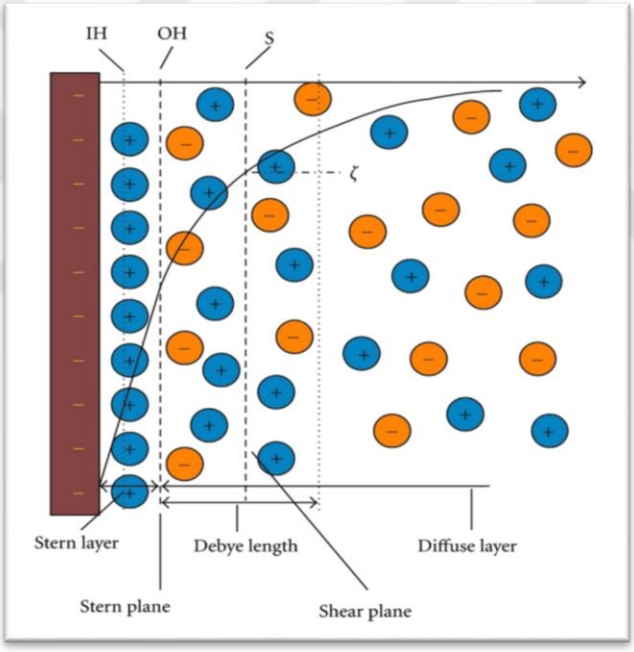

Figure 2.3 Electric double-layer according to the Stern model. The inner and outer Helmholtz planes are indicated as IH and OH, respectively. The slipping plane is denoted by S and its charge is characterized by the ζ-potential[54]

In electrochemical reactions, current is conducted from electrode-solution interface in two ways which are faradaic and capacitive. Firstly, while oxidation would be in one of the electrodes, reduction occurs in the other electrode. Therefore directly electron transmission is meantime obtained the current conduction. On the grounds that it is accordance the Faraday rules which says that the amount of chemical reactions in one electrode is proportianal to magnitude of current flow, current after redox reaction is

referred to faradaic current (i_f). Working electrode which is negatively charged and immersed in solution attracts cations and this causes occuring the voltage difference on formed interface. Opposite ions creates an electrical double layer which employs as an capacitor. There would be a current to charge the capacitor regardless of that redox reactions occured. This current which is independent from reaction, generated from the electrochemical system is called capacitive current (i_c). If i_c would be as small as possible, assasment accuracy is more powerful and sensitive[51].

Electrochemistry has an important role in the research and development of biosensors. In biosensor application tree-electrode system is commonly used[4-9] with carbon pasta electrode, glassy carbon electrode, gold electrode and screen printed gold/carbon working electrode. Various modifications of working electrode have been studied in biosensor application for effective bioelement immobilization on working electrode[10], amplification of biosensor signals[11-14], increasing sensitivity of biosensors[15-18], decreasing response time of biosensors[14], [19].

2.2 Cyclic Voltammetry(CV)

Electroactive material behaviors are analysed by examining oxidation, reduction, electron transfer mecanism, adsorbtion on electrode surface. Cyclic Voltammetry(CV) is one of the electroanalytic characterization voltammetry methods for electroactive material behavior analysis after electrochemical reaction at continiously changing applied potantial in unstirring solution and three-elecrode system. In CV, a definite variable voltage per second is applied by beginning from the determined first potantial value to finish potantial value at costant rate on working electrode. Voltage can be applied in negative or positive direction. Then the voltage is once again applied by beginning from finish potantial value to first potantial value at the same constant rate. Thus, reversible CV is obtained. Cyclic voltagram is constructed by varied current as a function of applied potantial. In CV, oxidative current which has been occured at positive applied potantial in oxidation is expressed negative sign. Obtained informations in CV characterization determine the optimum potantial which is appropriate to assess concentration gradient of substance in tree-electrode system. In figure 2.4, important parameters of CV are shown illustratively. In CV, important parameters are cathodic peak potantail ($E_{p,c}$), anodic peak potantial ($E_{p,a}$), cathodic peak current ($i_{p,c}$) and anodic peak current ($i_{p,a}$). If difference between oxidation and reduction potantial (ΔE_p) is as

much as small absolutely, it is reversible; if not, it is irreversible. In reversible CV, scanning times can be changed and then observing current changing helps interpretation of reaction parameters.

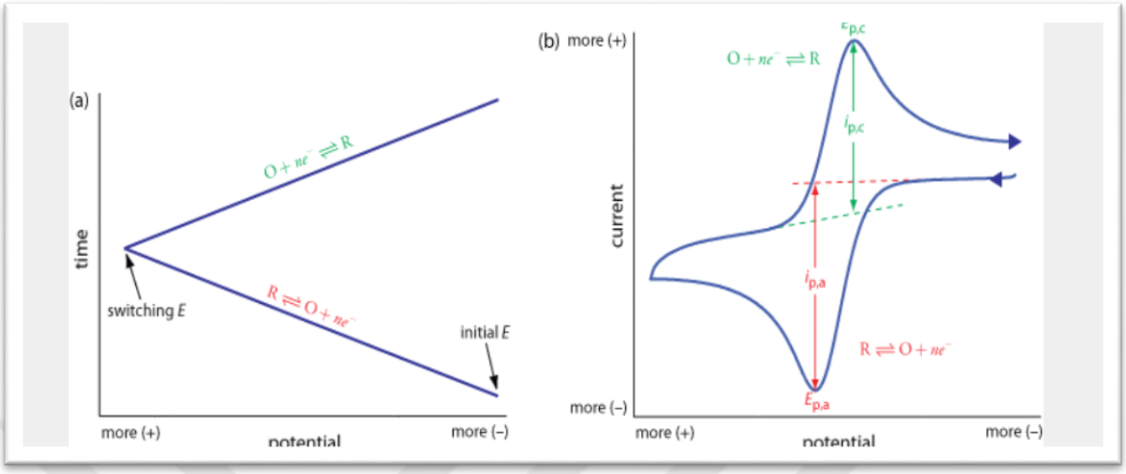

Figure 2.4 Typical reversible Cyclic Voltagram where O; oxidized, R;reduced, e^-; electron, $i_{p,a}$; anodic peak current, $i_{p,c}$; cathodic peak current, $E_{p,a}$; anodic peak potantial, $E_{p,c}$; cathodic peak potantial

In Figure 3, a typical CV diagram implies that oxidized substances (O) take electrons from cathode and are converted to reduced substances (R). It is illustrated a peak on voltamogram, and the peak height increases as scanning rate is increased. Top of the peak presents $E_{p,c}$ at when it is reached maximum current ($i_{p,c}$). Because R would be adequately found on electrode surface, R will oxidize to O at beginning from E_0 potantial to continously increased towards positive potantials in reverse direction. Thus, the peak of maximum oxidation current ($i_{p,a}$) is constructed at $E_{p,a}$. During reverse scanning, it would continue to arise R substances as reducing O substances until E_0 potantial. In reverse scannig according to Nernst equation, R surface concentration will decrease as potantial goes to more positive value and R will finally reach 0 at sufficient positive potantials. However, as a result of accumulated R substances on surface diffuse to solution, $i_{p,a}$ will be smaller than $i_{p,c}$[55].

A reversible electrode reaction at 25 ^oC formulated by Randles-Sevcik equation (2.3); where i_p is peak current, D_o is diffusion coeffiecient of O substance, C_o is concentration of O in solution and v is scanning rate. And E_p is not dependent to scan rate in reverse reduction. Relation of E_p and $E_{1/2}$ is shown in equation2.4.

$$(i_p)_{rev} = 2{,}69 \times 10^5 n^{3/2} A D_o C_o v^{1/2} \qquad (2.3)$$

$$E_p = E_{1/2} - 1.1\,\frac{RT}{nF} \quad (2.4)$$

CV peaks are effected by magnitude of current, concentration of electroactive substances, transfered electron quanification, surface area of working electrode and diffusion coefficient. One of the advantages of CV method is allowing to apply for varied potantial scanning rate. Thus, stability of redox reactions byproducts and possible another reactions which effect redox reaction can be analyzed[51], [56].

In biosensor applications, CV is used to analyze redox reaction kinetic and behaviour of electroactive substances in electrolyte solution in which an electrochemical interaction occurs between analyte (Ag) and immobilized bioelement (Ab) on WE. By CV method, whether electrode modification is successful and in parallel with this, whether modified electrode has conductivity and allows electroactive materials accumulation are informed. In CV method, various redox probes are utilised, those are commonly $K_3[Fe(CN)]^6$, dopamine, ferrocene, ascorbic acid. They perform electron transferring on electrode with high speed, reversibly. CV characterization by redox probe is fulfilled as comparing bare electrode voltamogram and modified electrode voltamograms[56-58].

2.3 Electrochemical Impedance Spectroscopy(EIS)

In 1886, "impedance" term is firstly used by Oliver Heaviside who is electrical engineer, matematitian and physician as adapting complex numerical system to electrical circuits[59]. In 1975, Lorenz and Schulze defined Electrochemical Impedance Spectroscopy (EIS) as measuring the resistive and capacitive properties of materials upon perturbation of a system by a small amplitude sinusoidal ac excitation signal typically of 2–10 mV[60]. EIS is a method analysis for electrode kinetics, double layer studies, batery studies, solid phase electrochemistry, the complex systems of electrical resistance, surface sensitivity and quantification of resistive, capacitive materials on surface. EIS is effectively and commonly used to analyse metal corrosion mechanism, charge transport through membrane and electrical characterization of surface/solution interface in the frequency domain. Double layer on electrode in an electrochemical system is where electrolyte ions accumulate as described in previous sections. The double layer interface employs as an integrated capacitor. A simple parallel capacitor is decribed with equation 2.5; where q is charge between layers at applied potantial difference, E is applied potantial and C is capacitance.

$$C = q/E \tag{2.5}$$

In electrochemical cell, thickness of double layer is proportianal to applied potantial difference. This capacitance can be measured by alternating current (ac) impedance spectroscopy. By ac-EIS, sinusoidal signal perturbation is applied to electrochemical cell and current change is analyzed. Sinusoidal perturbation is showed in equation 2.6; where V(t) is applied potantial at t, V_0 is amplitude of signal, and ω is angular frequency which is equal to *2πf*.

$$V(t) = V_0.\sin(\omega t) \tag{2.6}$$

Impedance is determined by applied potantial and ac current response which has same frequence, different phase angle and amplitude with applied potantial. Equation 2.7 implies general impedance expression; where *Z* is impedance, *V(t)* and *I(t)* is functions of potantial and current with respect to time, V_0 and I_0 are potantial and current value at maximum, *f* is frequence, *t* is time, *φ* is phase shift between *V(t)* and *I(t)*, Y is complex conductivity or admittance.

$$Z = \frac{V(t)}{I(t)} = \frac{V_0 \sin(2\pi f t)}{I_0 \sin(2\pi f t + \varphi)} = \frac{1}{Y} \tag{2.7}$$

Impedance has a vectoral magnitude which is expressed as Z(ω) and composed of a real (Z_R) and an imaginary part (Z_I). Z_I is out of phase component of Z which is related composition analysis of dielectric layers and Z_R is related reistance of system (Figure 2.5).

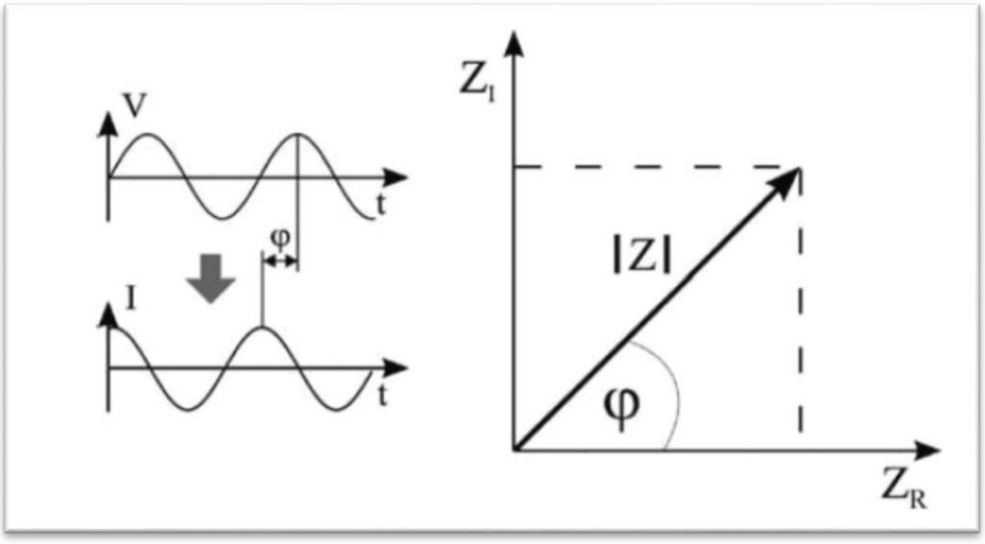

Figure 2.5 Expression of impedance with rspect to *V(t)* and *I(t)* function

Characterization of surface modification of transducers or membranes and information about varied physicochemical properties of these subtances are obtained using EIS method. To access this information, impedance spectrum is analysed with equivalent circuit which retains almost same electrical characterizations. This equivalent circuit is usually comprised with resistance and capacitance and used for aproximately determination of experimental impedance data by ideal impedance setting parallel and serial curcuit (Figure 2.6). This method has been using in electrochemical characterization system, generally. Impedance spectroscopic assesments are illustrated to analyse as bode plot which is graph of the frequency response and Nyquist plot which is a parametric plot of frequency response. Bode plot of EIS determines polarization resistance (R_p) at low frequency. Nyquist plot is drawn as plotting the real part on the horizontal axis and the imaginary part on the vertical axis. In this plot, the vertical axis is negative and each point on the Nyquist plot is the impedance at one frequency. In the figure 2.6, low-frequency perturbation (high energy) are on the right side of the plot and higher frequencies (low energy) are along the left. EIS model a process of diffusion toward or away from the surface with equavalent circuit (Figure 2.6). Z_I and Z_R represents imaginary part and reel part of impedance, respectively in Nyquist plot with equavalent circuit in figure 2.6 and they formulated in equation2.8, 2.9 and 2.10, where R_{SOL} is resistance of solution, R_{CT} is charge transfer resistance, k is chemical reaction rate, D is diffusion coefficient

$$Z_R = R_{SOL} + R_{CT}\left(1 + \frac{\lambda}{\sqrt{2\omega}}\right) \quad (2.8)$$

$$Z_I = \frac{R_{CT}\,\lambda}{\sqrt{2\omega}} \quad (2.9)$$

$$\lambda = \frac{k}{\sqrt{D}} \quad (2.10)$$

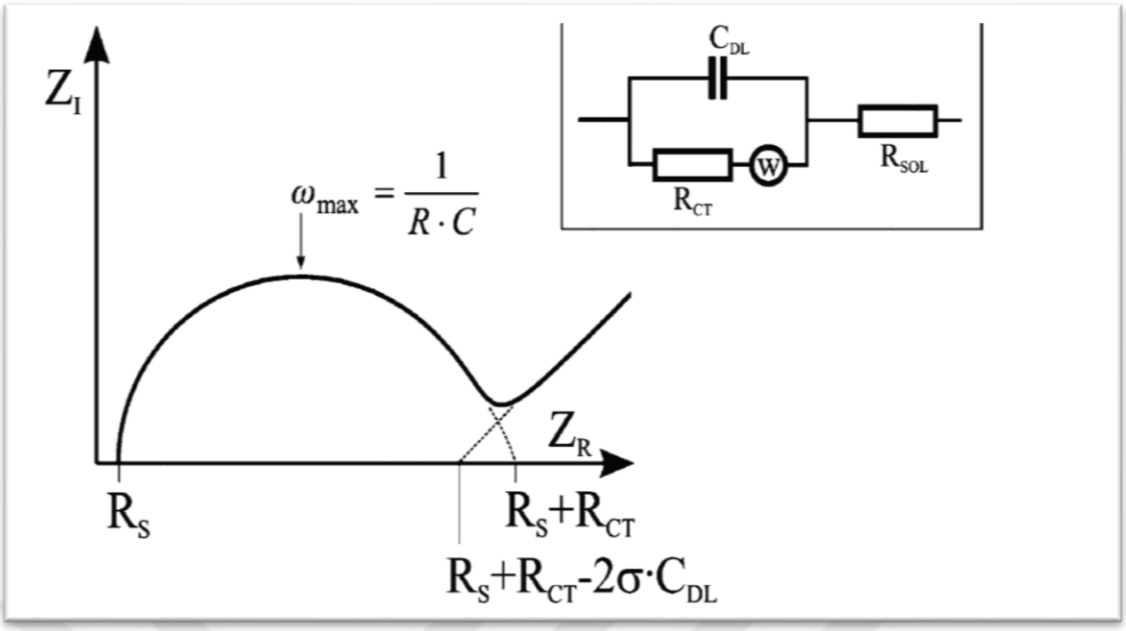

Figure 2.6Theoritically ideal Nyquist plot of Z(ω) and Randles equivalent curciut of EIS

In EIS there are four factors to explain impedance concept in electrochemical sytem in electrolyte solution which are ohmic resistance, capacitance, phase constant and Warburg impedance (Z_W). In EIS, equivalent curcuit is Randles curcuit includes solution resistance (R_{SOL}), charge transfer resistance (R_{CT}), double layer capacitance (C_{DL}) and Warburg impedance (W). Semicircle diameter of EIS Nyquist plot implies R_{CT}. Capasitance value of capasitor is at maximum Z_I value of spectrum. Linear part of the spectrum at which scanned low frequency is related to mass transfer between solution and electrode surface.

$$Z_W = \sigma(\omega)^{-\frac{1}{2}}(1-j) \qquad (2.11)$$

In equation 2.11, Z_W is warburg impedance, σ is warburg coefficient. Z_w is serial bond to R_{CT} showed in Randless circuit (Figure 2.6) and it shows the frequency dependent diffusive transport to electrode. At high frequency, $R_{SOL}$is measured and at lower frequency, energy gradually increases scanning moves to electrode surface. At the lowest frequency and the higest energy, electromagnetic waves go through electrode and create linear part of Nyquist plot which is related diffusion of substances on electrode.

Impedance is expessed in equation 2.12 based capacitance; where Z is impedance, R is component resistance, C is capacitance.

$$Z^2 = R^2 + \frac{1}{(2fC)^2} \quad (2.12)$$

In the term of impedance change, it is evaluated as quantification change of components at electrode-electrolyte interface. Electron transport at the interface occur from or to charged or uncharged atomic species in the electrolyte. Interface properties can be modified to develop various biosensor applications. Therefore, it is commonly used in biosensor applications essipecially affinity biosensors based DNA hybridization and immunological binding. In affinity biosenors, when the target molecule bind to immobilized reseptor molecule on working electrode, working electrode modification impedance change occur and this change is correlated by target molecule concentration[58].

2.4 Biosensors

Measurement of the amount of biological compounds such glucose, urea, enzymes, hormones etc. is performed with biosensor which converts biological reaction with some chemicals or biomolecules to quantifiable signal that can be amplified, stored and displayed[1]. As an example of converting biochemical reactions to a signal; an enzymatic reaction changes the current flowing through a conducting metal or polymer transistor[61]. Basic parts of biosensors are biological material, transducer and detector. Firstly, biological material (bioelement, biological recognition element) part has a sensitive selectivity to analyte which is measured biological compound. This part is immobilized to transducer against enviromental conditions and for getting efficient interaction between analyte and reseptor. Biological materials can be organelles, tissue, enzyme, antibody and so on. Secondly, the transducers are the parts of biosensors that amplify and control the input signal. Third part of the biosensor is the detector which shows the results as a quantifiable signal; this is the electronic part in the device. Figure 2.7shows the general schematic of a biosensor. The interactions between analyte and reseptor molecule occurs physical, chemical or electrical changes on transducer part of biosensor. The changes became after these interactions can be exemplified as increasing or decreasing of electroactive materials, changing of temperature, light, mass, pH and viscosity[1]. There is a correlation between these changes and concentration of analyte and transducer par of biosensor transmits this correlation to electronic parts of biosensor as quantifiable signal. This signal is an interpretation of concentration of analyte. So

that, diagnosis and treatment of diseases could be getting fast, easy and economic after analyzis of concentration of biomomolecules/analytes by using biosensors[57], [62].

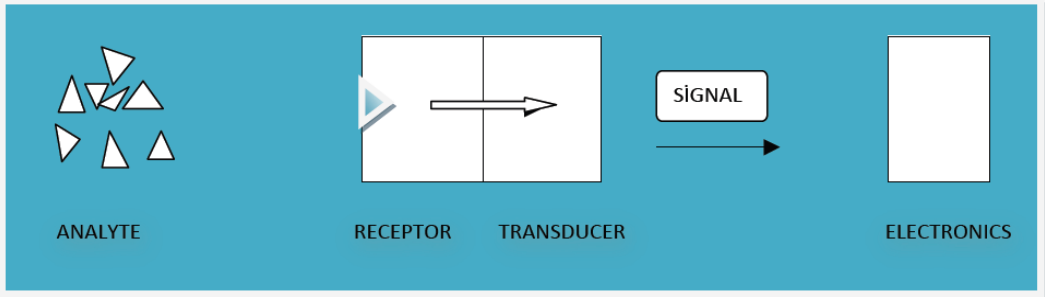

Figure 2.7General schematic of a biosensor[63]

In 1962, the first biosensor was used for the determination of glucose concentrations in blood samples by using an enzyme-coated oxygen electrode[64], [65]. Lenald C. Clark placed glucose oxidase enzymes on Platinum electrode by help a membrane. Glucose and oxygen molecules are converts to gluconic acid and peroxide through glucose oxidase enzyme (Equation 2.13). After chemical reaction between glucose and oxygen molecules, electrons pass through Pt electrodes (Equation 2.14). It is provide that quantifiable signal to read concentration of glucose molecules in oxyganated medium because passed electrons are correlated with glucose molecule concentration[24], [64], [65]. Glucose biosensor which detect glucose from one drop of blood were developed later on. To provide a standardization of oxygen-peroxide conversion, oxygen level has to be retained in a stability. So that an mediator molecule inserted in the oxygen-glucose medium[21]. Articles about biosensors started to become widespread in the beginning of 20. Century. In the beginning period of BioSensing research-development activities in 1955-56, Clark investigated oxygen electrode and introduced glucose biosensor[65-67]. With the innovation of biosensors after that time, various diagnostic tools and emergency tests have been improved and became widespread[61], [62].

$$glucose + O_2 \xrightarrow{glucose\ oxidase} gluconic\ acid + H_2O_2 \quad (2.13)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (2.14)$$

There is a similarity between sensing of small molecules system in our body and biosensor working mechanism. For instance, sense organs perceive small molecules around our environment, transmit the signals to brain and then; after interpretation of signals by brain, the body gives responses. From this point of view, if sensing systems in our body are scrutinised, there would be an important breakthrough in the

development of biosensors. For biosensor researches, material science and chemistry have also very important place together with science of molecular biology. In terms of bioelement immobilization on transducer, material scince and chemistry have an important place. Also there is some way to produce artificial bioelements by chemistry. In conclusion, biosensors are constituted and developed by multiple disipline like molecular biology, chemistry, analytical chemistry, nanotechnology, material science, electronic and so on.

There are some main characteristic and critical points of an ideal biosensor. They are selectivity, stability, sensitivity, reusability, regenerability, reproducibility, lifetime, calibration, limit of detection, measuring range, response time, easiness, economic, sterilization and miniaturization. Selectivity is the one of the most important parameter of an ideal biosensor. Reseptor/biomolecule which is immobilized on transducer surface has to be selected highly specific to analyte/target molecule. If selectivity of biomolecule is weak, there can be nonspesific interaction between biomolecule and some substance unwanted detection, so that interfering signal can ocur and accuracy of biosensor is influenced. One of the factor limits lifetime of biosensor is decrease of biomolecule activity. This also effects other parameters like calibration interval, stability, reproducibility. The need of calibration has to be less or there has to be any calibration for an ideal biosensor in theoritically, but in practical this is difficult. So that biosensors are frequently calibrated till end of the life time. For an ideal biosensor, if used electrode for measurement are repeatedly being used in the measurement of analyte after sterilization of the same electrode, it would be expected to have almost same or near results. Whereas this is an unlikely situation in practical studies, reproducibility parameter of biosensor has to be investigated well enough. As reproducibility has a good quality so biosensor performance will be successful. High stability of an electrode/transducer of biosensor is a necessary for an ideal biosensor. Stability is related to physcical endurance of bioelements on transducer. It is also affected from parameters like pH, temperature, humidity, ion concentration and other enviromental effects. Limit of detection of analyte concentration has to be small values and this parameter is affected from area of electrode surface, affinity between bioelement and analyte and amount of biomolecules which will be immobilized on electrode or any transducer surface. Detection range of analyte concentration has to be a wide interval for a successful biosensor application. Another intended property is being

a lineer relationship between signal of biosensor and analyte concentration. Response time of a biosensor has to be as short as possible to make a fast measurement. Easy fabrication and more economical design of biosensor is preffered properties. Sterilization parameter is also important for an ideal biosensor. It is affected physical endurance of bioelements on electrode/transducer[1], [21], [57].

2.4.1 Immobilization Methods of Bioelement

Immobilization of bioelement on transducer surface of biosensor has a critical role in a biosensor fabrication progress. In biosensor applications, immobilization is to increase bioelement stability on transducer surface for increasing efficiency of physicochemical interaction between bioelement and analyte. Adsorption of bioelements on transducer surface is basic and easy method in the immobilization treatments. Adsorption is binding of molecules or particles on surface. Microencapsulation is another method in immobilization and it is also first used among others. In microencapsulation, bioelements are covered with a semipermeable membrane so that they are got more immobile on transducer surface. Entrapment is an immobilization method which provides to stabilize bioelements with gel matrix or polymer matrix. In this method, bioelements are stuck into the gel matrix or polymer matrix. Covalent binding of bioelements on transducer surface become with a covalent bond between functional group of bioelement and modified transducer surface with molecules could bind functional group of bioelement. This is also an immobilization method. Cross linking is another method which is provided in conjugation with microencapsulation method and adsorption method[1], [21].

Enhancements in immobilization techniques were observed with the nanotechnological developments. Researchers in their works implied that nanomaterials increase surface area of transducer surface which is covered with them and so that sensitivity of biosensor increase considerably[10], [22], [23]. By using nanomaterials and organic molecules in transducers of biosensors, we have an opportunity to their miniaturization and integration into portable electronic devices for medical applications of biosensors[24]. The important factor for enhancement of immobilization yieldance is that using nanomaterials during surface modification. Nanomaterials like nanoparticles, nonotubes, nanowires enhance immobilization yieldance and so biosensor yieldance because of their high surface-to-volume ratio and novel electron transport properties.

High surface-to-volume ratio provides larger number of antibody immobilization on transducer and their electronic conductance is strongly influenced by small change on transducer (antibody-antigen binding), hence indicating high sensitivity and great promise for label-free real-time protein detection. Using nanomaterials in biosensor fabrication also indicate great promise for multiple disease markers assasment in ultrasmall sample volumes[33], [68].

2.4.2 Classification of Biosensors

When examined the biosensor literatures, there are three types of biosensor denotation based on bioelement, transducer type and analyte. In the fabrication of biosensor, type of immobilized bioelement, type of trancducer type and measured element as an analyte determine whole name of biosensor. In the naming of biosensor, more than one type can be used together. For instance; the denotation like “Glucose biosensors based on carbon nanotube nanoelectrode[69]” is based on analyte type and transducer type together denomination. “Enzymatic biosensors based on carbon nanotube-conducting polymer electrodes” is based on type of immobilized bioelement on transducer and type of transducer denomination[70]. In terms of classification of biosensors, bioelements and transducers are focused in literature[1], [2], [21], [57], [71]. Figur 2.8 shows that classification of biosensors. Biomolecule based biosensors are enzymatic biosensors, immunosensors, nucleic acid based biosensors, microbial biosensors and carbohydrate biosensors. Transducer based biosensors are electrochemical, optical, acustic, thermal and microcantilever biosensors.

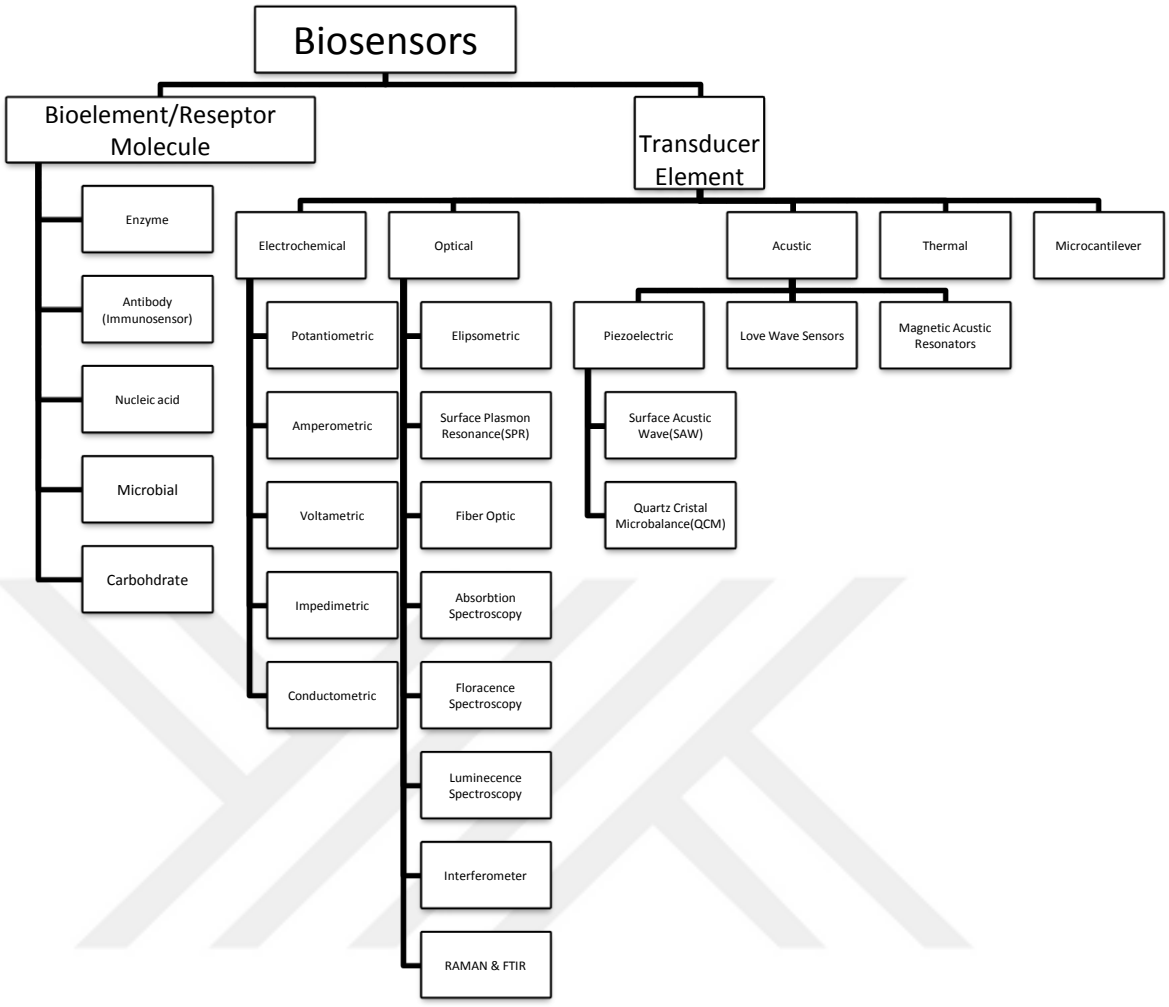

Figure 2.8Schematic of biosensor classification[1], [2], [21], [57], [71]

2.4.2.1 Classification of Biosensors Based on Bioelement

2.4.2.1.1 Enzymatic biosensors

Enzymes are employed in enzymatic biosensor application as bioelement which catalyzes electroactive substances production on transducer of biosensor to determine concentration of interested analyte. Their usage in biosensor application is quite widespread because of high sensitivity and specificity properties of enzyme molecules. Enzyme immobilization on transducer of biosensor is important step in fabrication of enzymatic biosensor. Because enzymes have high biocatalytic activity and specificity, enzymatic biosensors are commonly being developed with various electrochemical transducer. Enzyme electrodes among electrochemical transducers are commonly used to product enzymatic biosensors to detect monosaccharides, amino acids, proteins,

hormones, organic acids (lactic acid), urea and alcohol. Enzyme electrodes produced with a thin layer of immobilized enzyme on its working electrode. Enzymes catalyze electroactive component production for detection. The detection of electroactive material decreasing or increasing is commonly determined by amperomeric transducer, in which the produced current is measured in response to an applied, constant voltage. Electrode design and electrolyte solution parameters effect the activity of immobilized enzymes. Enzyme stability and shelf life determine the lifespan of the enzymatic biosensor. Advantagous of the use of enzyme electrodes is inexpensive and simple fabrication, reusable, fast response and regenerable. However, immobilized enzyme on electrode has to be replaced periodically since it gradually loses activity. This disadvantagous shows that stability parameters of these type biosensors are weak according to other parameters[3], [72].

Common enzyme immobilization techniques are covalent attachment by functional groups of enzyme such as NH_2, COOH, OH and SH to transducer surface, cross-linking between enzyme and transducer, adsorbtion which is formed by primarily very weak van der Waals forces with occasional hydrogen bonds, entrapment using a preformed membrane, encapsulation of enzymes inclusion in a gel or electropolymerized film on transducer[73]. Encapsulation and cross-linking obtain more stable immobilization than other immobilization techniques[1]. In enzyme immobilization, it has to be attantive to form on the transducer surface without blocking active site of enzymes to maintain selectivity of biosensor[74]. Immobilization techniques have being developed with some nonotechnological materials such as nanotubes, Q-dots, thin films to produce more sensitive biosensors[75]. Delvaux M. et. al. studied on Glucose Oxidase(GOD) enzyme immobilization on gold nanotubes. Polycarbonate(PC) covered electrode is modified with gold nanotubes, this modification increases surface area of PC electrode. After this modification, GOD is immobilizaed on gold nanotubes with help of a linker molecule which has thiol group (-R-SH) for linking to gold nanotubes by SH functional group and to react with amineor carboxyl group of enzyme molecule by R functional group. In that study, importance of nanomaterials are focused because increasing surface area of electrode provides high sensitivity in comparision with non-nonamaterals[76].

2.4.2.1.2 Immunosensors

Immunosensors are biosensors have fabricated with antibody immobilization on transducer. Bioelements of these biosensors are antibodies. These biosensors are developed with prominent properties of an antibody(Ab) that are specificity and affinity to an antigen(Ag) causes diseases. These significant properties provide construction of the highly selective biosensor. Ab-Ag interaction generates physical or chemical changes on transducer which converts this change to quantifiable signals. Abs recognize and bind to Ags or haptens by its specific binding reagions (complementary regions). Immunosensors had been constructed by different type of transducers which are electrochemical, optical, thermal, acustic and microcantilever. Most of the developed immunosensors are based optical and electrochemical transducer. In this section, Ab structure and production will be elaborated, important points in terms of the fabrication of an immunosensor and some other immunoassesments will be mentioned.

2.4.2.1.2.1 Antibody Structure

Antibodies (Ab), which involve large Y-shape glycoprotein molecule, are produced by a host in response to the presence of a foreign molecule (antigens) in specialized B lymphocyte cells of the immune system. Abs can usually be found in blood serum, tissue fluids, and membranes of vertebrates. Examples of Ags can be chemical compounds, proteins, dust, pollen, bacteria, virus which the body recognizes as foreign. Abs are included in blood as immunoglobulin proteins abreviated “Ig”. Immunoglobulins are composed 20% of total human blood serum proteins. Immunoglobulins are dividied into 5 main groups; those are IgG, IgM, IgA, IgD and IgE. This classification is based on variation about dimention, charge, aminoacid components, carbohydrate components of glycoproteins[77]. IgGs are widely used for therapeutic purposes and in this thesis, IgG for carcinoembriogenic antigen was used so IgG structure will be detailed.

IgGs constitude 80% of all immunoglobulin protein types in human blood serum and play an impotant role in fight against viruses. Four polypeptide chains constitude the IgG molecule; two of them are heavy chains with MW of 50 000 and two of them are light chains with MW of about 25 000, identically.Heavy chains (H_C) and light chains (L_C) that are held together by disulfide bonds and noncovalent interactions such as hydrogen bonds as seen in Figure2.9.The stem of the Y form of antibody is a constant

region composed of only heavy chains with carboxyl functional groups ends. Each Ab molecule has two identical antigen binding sites which is highly specific for related antigen and formed at the tips of each of the Y arms of antibody.The ends of the arms of the Y form antibody has variable regions composed of heavy and light chains which are variable region of light chain (V_L),variable region of heavy chain (V_H) and contribute to the finger like loops that interact with the antigens.This variable region binds to the specific antigens. Fragment regions (F_R) provide stability to the anitbody and when folded into seconday structure. Constant regions of heavy chain(C_H) is used to interact with effector cells and complement. Complementary Determining Regions (CDRs)differ most in their sequence and structure between different antibodies.The hinge region is a segment of the C_H chains and critical for the flexibility of the Ab.

The noncovalent interaction between the Ab and Ag is highly specific with an irreversible chemical alteration. These noncovalent bonds are hydrogen bonding, ionic bonds, hydrophobic interactions, and van der Waals forces. The unique antibody-binding region of the CDR binds highly selectively with high affinity to a complementary site on the antigen. The binding of Ab to an Ag is also very powerful and affinity.

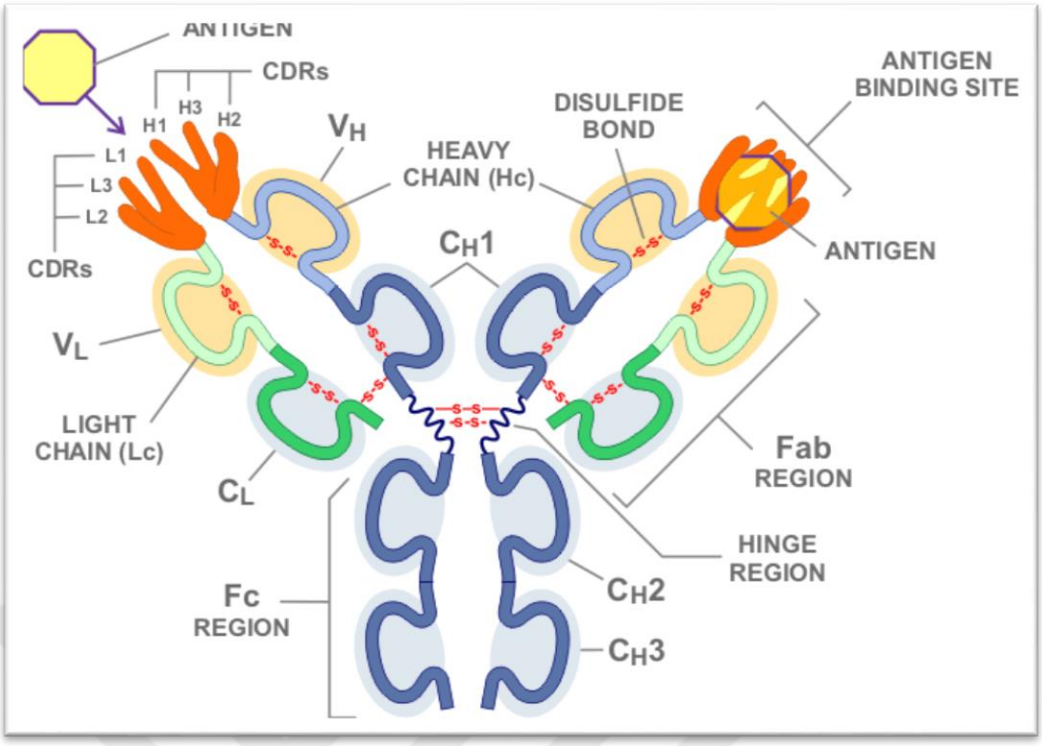

Figure 2.9Y-shaped antibody structure. H_C, heavy chain; V_H, variable region of heavy chain; L_C, light chain; V_L, variable region of light chain; $C_H^{1,2,3}$, constant regions of heavy chain; C_L, constant region of light chain; CDRs, Complementary Determining Regions; $H_{1,2,3}$, CDR loops per variable domain on the heavy chain; $L_{1,2,3}$, CDR loops per variable domain on the light chain; F_C, fragment crystallisable; F_{ab}, fragment antigen binding[78]

2.4.2.1.2.2 Antibody Production

Antibodies had started to used for diagnostic and identification tool since 1950 in various field such as; enviromental issues, clinical diagnostics, defence industry[79]. In immunosensor applications, rabbits, mice and goats Abs are commercially, commonly used with polyclonal or monoclonal production in host organisms (rabbit, mouse and goat).Antibodies specific for an antigen of interest can be used as immunodetection tool for antigen in biosensor applications. The production and use of specific antibodies for antigen detectionhas very impotant place in bioresearch and diagnostic technologies. Antibody production are based that animals immunized with prepared antigens will produce specific antibodies against the antigen. When purified from serum or hybridoma cell lines, the antibody can be used directly or after labelling in immunodetection applications. Antibodies are most commonly purified by one of two affinity purification methods: general immunoglobulin purification or specific antibody purification. The production of the Ab against a specific Ag can be difficult and time-

consuming. Monoclonal Abs are homogenous molecules against to specific antigens which recognized by an epitope of Ab. In monoclonal Ab production, mice, microbial systems and transgenic mice are being used with high specificity and affinity as the primary or capture Ab in most research, diagnostic, and sensing applications. Polyclonal produced Abs are a heterogeneous mixture of immunoglobulin molecules secreted against a specific antigen, each recognizing a different epitope, so that antigen affinities can be vary and are often used as the secondary Ab in immunoassays[80].

By using the production of Abs in host organisms, they can be diversify for a wide range of substances, species such as biomarkers, bacteria, viruses, spores, drugs, hormones, pesticides, and numerous other chemicals[1], [81]. Theoritically, if an Ab is produced for against a particular substance, an immunosensor could be developed to detect for that substance with high selectivity and sensitivity having low detection limits i.e. detecting trace levels (ppb, ppt) of analytes[3]. Because of potantially detectable analyte variety, applications of immunosensors have a wide range in using areas which are food safety, detecting enviromental pollutants, detecting biomedical substances and biological pathogens. Immunosensors are using to develop diagnostic kits such as pragnancy and fertility tests, and in this context, immunosensors are attractively developing to produce fast, cheap kits and provide ease of use in medical[40].

2.4.2.1.2.3 Conventional Immunoassays

Immunoassasment have been conventionally provided by enzyme linked immunosorbant assays (ELISAs) and radio immunoassays (RIAs) contributing advantages of high selectivity, high affinity of antibody molecules and quick, sensitive response. But there are also some problematic issues in these methods like radiation hazards, time consuming, qualified personel and sophisticated instrumantation[58].

Enzyme immunoassays were developed as an alternative to radioimmunoassays. Radioactive labelling replaced by a safer, selective and less expensive enzyme label at the cost of less sensitivity and more complexity. Enzyme labelling of Abs generates electroactive products after Ab-Ag interaction. Changes amount of electroactive products give information about Ab-Ag interaction by electrochemical signals. Enzymes are also highly selective for their given substrate, and can provide a large signal amplification.One of the biggest disadvantage of enzyme immunoassays is that the activity of the enzyme labels can be affected by reaction conditions. Like

radioimmunoassays, enzyme immunoassays can be time-consuming due to laboratory procedures[79].

Enzyme linked immunosorbent assays (ELISA) is an enzymatic immunoassay methods and leads to the formation of a sandwich complex (Primary Ab/Ag/Secondary Ab). Procedures are; first, the Ag sample is incubated with an excess of Ab reagent. All the Ag molecules form a complex with antibodies, but not all of the Ab-binding sites are occupied. To detect the amount of Ag attached to an Ab, a labeled secondary Ab is added which binds to another, available epitope on the bound Ag. Finally, unbound excess reagent is washed away after each incubation step. End of the electrochemical reactions, there will be a quantifiable signal to interpret Ag concentration. Compared with conventional immunoassay methods, electrochemical immunosensors gets some important advantages such as low cost, ease of use, fast analysis, portability, miniaturazation, small analyte volume and simplicity of fabrication[82].

2.4.2.1.2.4 Fabrication of an Electrochemical Immunosensor by Transducer Surface Modification

Electrode modification is crucial step in fabrication of an immunosensor for antibody immobilization on working electrode of electrochemical biosensors. Various mateials as mediator, amplifier or linker have been used in the electrode modification. Due to highly conductivity, increasing surface area of substance, allowing effective immobilization, labellig Ab to amplification electrochemical signal, nanomaterials, conductive, semiconductive polymers or molecules have been favorable using in electrochemical immunosensor electrode modification (Table 2.1).

Tablo 2.1Commonly used materials in an electrochemical immunosensor electrode modification

Structures	**Materials**	**Properties/Purpose**	**Reference**
Metalic nanoparticles	AuNPs, PtNPs, AgNPs	To increase surface-volume ratio, conductivity	Ref[32], [83]
Metal oxide nanoparticles	TiO_2NPs, MnO_2NPs	To increase surface-volume ratio, conductivity	Ref[84], [85]
Nanowires	SiNWs, CPNWs	To increase surface-volume ratio, conductivity	Ref[86], [87]

Table 2.1 (Cont'd)

Nanorods	Au Nanorods, ZnO Nanorods, Diamond Nanorods	To increase surface-volume ratio, conductivity	Ref[29], [88], [89]
Nanotubes	CNTs, SWCNTs, MWCNTs	To increase surface-volume ratio, conductivity	Ref[18], [90], [91]
Nano thin film structures	ZnO thin films, Graphene nanosheets	To increase surface-volume ratio	Ref[92], [93]
Magnetic nanomaterials	Fe_2O_3@AuNPs	Strongly paramagnetic, biocompatible /To employ effective carrier, To increase surface-volume ratio	Ref[5], [94]
Nanocomposites	Au/TiO_2, graphite/paraffin, AuNPs/polypyrole, Au/ployalinine Silver/polyphenylpyrrole, CNTs/polypyrole, TiO_2/ polythiophene, Fe_2O_3/polypyrrole, Fe_3O_4/ polypyrrole	To increase surface-volume ratio, conductivity	Ref[95], [96]
Conductive polymers/molecules	Poly(acetylene), poly(pyrrole), poly(thiophene), poly(terthiophene), poly(aniline), poly(fluorine), poly(3-alkylthiophene), polytetrathiafulvalene, polynapthalene, poly(p-phenylene sulfide), poly(o-phenylenediamine), poly(3,4-ethylenedioxythiophene)	Conductivity / To confirm matrix formation for Ab encapsulation, stability of immobilization	Ref[96-98]
Semiconductive polymers/molecules	Polystyrene, doped Si, poly(3-hexylthiophene), pentacene, poly(paraphenylenevinylene)-CdSe	Contrallable conductivity / To confirm matrix formation for Ab encapsulation	Ref[99], [100]
Nanoporous film	Nanoporous gold film	To increase surface-volume ratio	Ref[17]

Table 2.1 (Cont'd)

Hollow microshere	Gold hollow microshere (GHM)	To increase surface-volume ratio	Ref[101]

Magnetic nanomaterials constructed by Fe_2O_3, conjugation with nanomaterials. In addition to conductive or semiconductive polymers, mixed polymers layers are coated electrode surface to increase conductivity and to provide matrix for immobilization antibodies[102]. Refered studies imply that these materials can be obtain right orientation of antibodies with good compability and enhance sensitivity by improved ability of low concentration detection. Antibodies conjugated with nanoparticles using tailored protein units as linker, can be immobilized on electrode surface by microencapsulation with hydrophilic, porous, and positively charged alumina sol-gel matrix[85]. Above tabulated micro and nano materials amplifies biosensor transduction signals and are improved in assesments of antigens in PBS or real biomedical samples. In 2006, Yu et. al. reported a novel amplification strategy for SWNT immunosensors to the detection of prostate specific antigen (PSA) in real biomedical samples for the first time by measuring the current for catalytic peroxide reduction under a constant voltage. They attained detection limit of 4 pg mL^{-1}, for PSA in 10 µL of undiluted calf serum[18].

In addition to above mentioned advantages of nanomaterials, using nanomaterials as tagging for amplification of electrical bioaffinity with remarkable sensitivity and multiplexing capability is implemented various biosensor application. Examplifying this with a study published in 2015, amplification of electrochemical biosensor signal will be plainly understood. C. Zhang et. al. developed a sandwich-type immunosensor by Au working electrode modification with thiol aromatic aldehyde(which can be covalently bind Au surface and Ab_1, Fe_3O_4@Au nanoparticles and PAMAM) to bind IgG antibody on electrode surface. Up to the study of this part, antigens could be recognized by immobilized antibody, but electrochemical signals would be weak. Fe_3O_4@Au/PAMAM/Ab_2–HRP bioconjugated molecule has been synthesized as tagging Fe_3O_4@Au with polyamidoamine (PAMAM) and horseradish peroxidase (HRP) conjugated second IgG antibody to amplify signal after binding TAA/Ab_1/Ag modified electrode. Finally, a sandwich-type immunosensor developed with linearly detection range, 0.005-50 ng/ml, and limit of detection, 3 pg/ml by EIS, DPV and CV electrochemical characterization methods (Figure 2.10)[5].

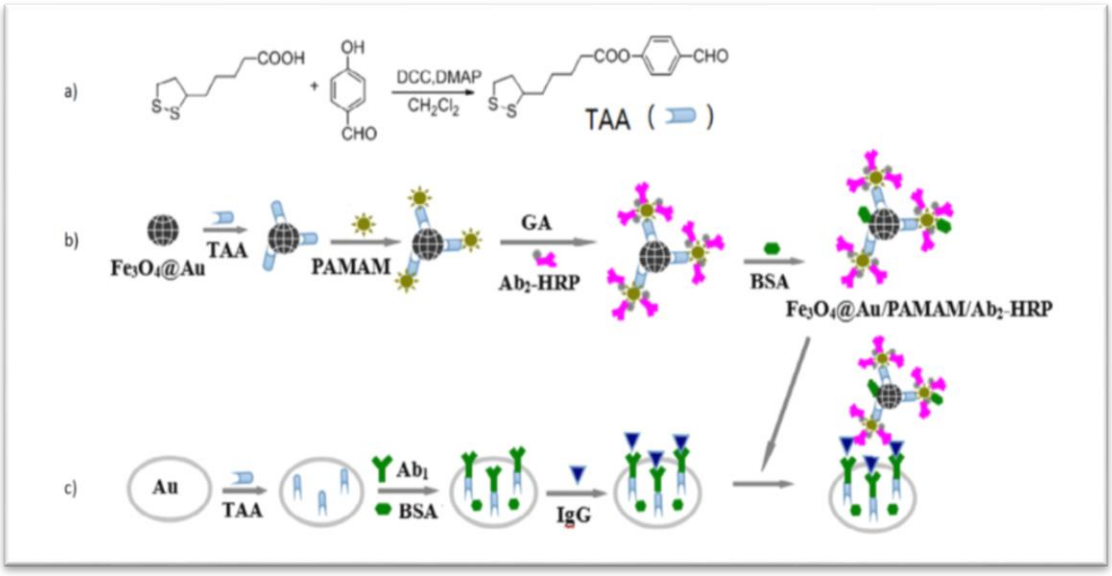

Figure 2.10 a) Synthesis of TAA b) Preparation procedure of Fe_3O_4@Au/PAMAM/Ab_2–HRP c) Immobilization process of electrochemical immunosensor (DDC; N,N'-Dicyclohexylcarbodiimide, DMAP; 4-Dimethylaminopyridine, TAA; Thiol aromatic aldehyde, PAMAM; Polyamineamine, GA; Gluteraldehide, HRP; Horseradish peroxidase)[5]

Gold materials are commonly used as biosensor transducer surface or in the transducer surface modification using gold nanoparticles with various shapes[29], gold nanorods and various composite with gold nanomaterials because gold materials have a number of desirable properties which are essentially inert, non-toxic and biocompatible. Gold layer obtains easy adsorption of biomaterials relates to hydrophobic and thiol–gold interactions during immobilization process[30]. In recent years, gold nanoparticles (AuNPs) are commonly used to enhance more sensitive electrochemical immunoassay for immobilization of antibody. AuNPs provide strongly adsorbtion of antibody on working electrode during immobilization due to its large specific surface area, good biocompatibility, surface free energy of nanosized particles[31], [32]. AuNPs facilitate electron transfer between redox proteins and electrode surfaces, provide effective mass transport in electrochemical biosensor applications as making closer redox protein (Ab) to electrode via nanosized structure. In the other words, AuNPs is a desirable intermediator for immobilization of bioelements[33].

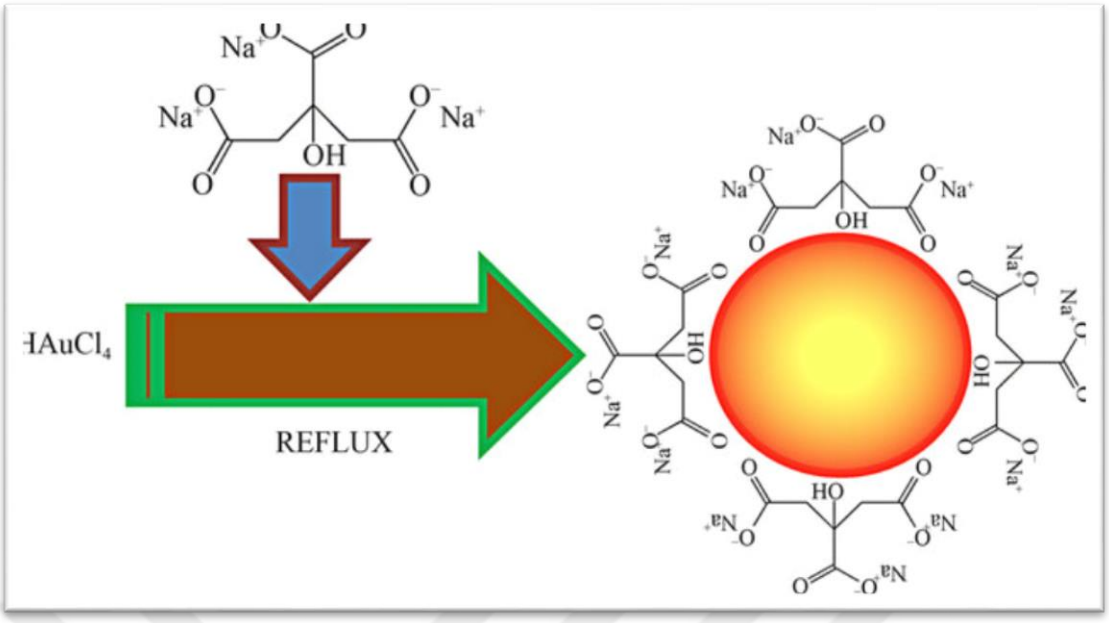

Figure 2.11 Gold nanoparticle stabilization with trisodium citrate[103]

Colloidal AuNPs solution as biolinker have been extensively prepared for immobilization of antibodies to costitude AuNPs layer on thiol modified working electrode[34].Synthesised AuNPs by Frens method produces AuNPs with reducing gold ions in $HAuCl_4$ by citrate molecules which is schematized in figure 2.11. The reduced gold atoms in solution, aggregate and form clusters, these clusters grow by increasing the number of atoms until achive the particles form. In AuNPs synthesis, trisodium citrate acted as a capping agent and thus restricted the growth of Au[47].

Thiol layers are significants and extensively being used to modify metal surface asspecially gold surface because of extremely high affinity between gold and sulfur atoms. Adsorbtion of molecule with thiol can be constructed by Self Assamble Monolayer (SAMs) technique[35]. In recent years, there is mixed self-assembled monolayers because construction of mixed SAMs interfaces by co-adsorbing thiols of different chain length or end group functionality provide a controlled chemistry and structure. Mixed SAMs using alkanethiols of different chain length or end group functionality increase antibody immobilization efficiency by enhancing functional surface area and reducing reducing steric hindrance [36]. Modification of working electrode with collaidal AuNPs helps oriantation of proteins on gold nanoparticles conveniently. AuNPs morphology and distance between particles are important factors which at connection between working electrode and redox proteins. Blocking reagents are employed to block the possible remaining active sites of the nano-Au monolayer,

avoid the non-specific adsorption of antibody in immobilization process and so they amplify the signal response of the antigen–antibody reaction on electrode. Commonly used blocking reagents on nano Au layer are BSA, HRP and ethanolamine[8], [37-39].

In process of the immunosensor fabrication, Ab immobilization has a critical point to obtain selectivity and sensitivity of biosensor. To bind Ab on electrode surface, there have to be surface modification by various mateials and maintance Ab binding on electrode with right oriantation. If the oriantation of Ab on transducer surface can not allow the binding to Ag, desired immunosensor design could be unsuccesful beause of loss of biochemical interaction of Ab-Ag as increasing background signal. Complementary antigen binding sites of immobilized antibody have to be allow efficiently binding as well as to antigen and therefore Abs cannot be randomly oriented on the surface. It is named nonspecific binding(NSB) which involves the adsorption of conjugated enzyme or other labels used for immunoassay to materials other than the analyte[40].There are enhanced methods to reduce nonspecific interactions between immobilized Ab and Ag; which is using a nonionic surfactant, Tween 20 and bovine serum albumin (BSA), polyethylene glycol, gelatin, casein as protein blockers of nonspecific binding region of antibody during immobilization[91]. As using blocker, minimizing NSB is also important for determination of detection limit and accuracy of immunosensor. Prevention of NSB is implemented by (SAMs) of oligo(ethylene glycol) and dextran layers have also been used successfully in surface modification of transducer.

Thomas D. J. et. al. immobilized anti-8 hydroxyguanosin mouse monoconal antibody on Silicon nanowire(SiNW) to produce a biosensor. Purpose of this biosensor is detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG) biomarker which is released from cancer cells because of DNA damage on their relative gene. In this study, immobilized monoklonal antibodies provide a resistance on SiNW of Field Effect Transistors(FET) which is transducer part of biosensor. When biomarkers interact monoclonal antibodies, resistance on SiNW increase with a correlation of biomarker concentration. Linking groups include $–NH_2$ group provide binding to –COOH group of antibodies and increasing signal amplification as in like that study[104]. Immunosensors are also known as affinity sensors because of having highly selective bioelement i.e. antibody.

In this study, an electrochemical impedimetric immunosensor was developed for detection of CEA as a cancer biomarker. In construction of immunosensor, SPGE was

thiolated with 1,6-hexadithiol then AuNPs were covalently attached with thiol group of 1,6-hexadithiol. Monoclonal anti-CEAantibodies were immobilized on working electrode of SPGE modified with AuNPs. BSA was used as blocking reagent. Electrochemical characterization was implemented by CV and EIS. Synthesized colloidal AuNPs were characterized by FESEM morphologically.

2.4.2.1.3 Biosensors based on nucleic acids

Biosensors based on nucleic acids are using for detection of specific DNA sequences. Bioelements of these biosensors are single stranded oligonucleotide that allows hybridization of its spesific strand which is the analyte of interest. In these biosensor application, nucleic acid chain hybridization is generally observed by optic or electrochemical transducer. Nucleic acids are now becoming of greater importance as the biorecognition agent in biosensor applications ever since there is an increased studies on DNA molecule and artifically production of replicated DNA affinity probes. DNA hybridization biosensors are also known affinity sensors because of highly selectivity of DNA probes as bioelement. DNA affinity probes are typically used in medical diagnostics to detect mutated DNA sequence and so to diagnose genetic diseases, viral infections or other infectious diseases which damages DNA, for the elucidation of mechanisms of drug interaction in the organism and in forensic medicine[1], [56]. DNA probes can be also used in determination microorganism contamination because a gene of microorganism responsible for specific property can determine by DNA biosensors[56].

In biosensor based nucleic acids fabrication, 20-40 basepair single-stranded DNA segments which is specific to target analyte are immobilized on transducer surface. In immobilization process, there have to be attantion to retain stability, reactivity and optimal oriantation of DNA fragments[56]. In electrochemical transduced nucleic acid based biosensors, an electrical signal gives information of anayte target concentration with help of electroactive indicotor after target DNA binds to the complementary sequence. The electroactive indicators and intercalators can binds to either the DNA duplexes or single-stranded DNA probes suc as ferrocenyl naphthalene diimide (FND), Meldola's blue (MDB) and provides intraction to specific nucleic acid base or intercalate DNA strand to increase electrochemical activity and amplify electrochemical signal[105], [106]. For electrochemical measurements, enzyme labelling to DNA probe

produce electroactive materials by redox mediator like polymer, $Ru(bpy)_3^{2+}$ and increase electrochemical signal amplification after hybridization. HRP and alkaline phosphatase can be as examples of those enzymes[107], [108]. In electrochemical nucleic acid based biosensors, magnetic particle, collaidal gold nanoparticle labeled DNA probe have also been developed in DNA biosensors applications to amplify electrochemical signal and increase electroactivity[51], [108]. Conductometric and impedimetric transducers of electrochemical biosensors allow that improving label-free detection of target DNA probe with changing conductivity and resistance after hybridization on transducer[56].

In optical transduced nucleic acid based biosensors, quantum dots are extensively used. Gua X. et. al. developed a nucleic acid based optic biosensor by using quantum dots. In that study, principle of biosensor is that hybridization of DNA strands which are specific to each other provides different wave lenght and fluorescence intensity on spectroscopy with help of quantum dots. DNA strand are immobilized on quantum dots and after hybridization optical analysis are possible[109]. As using this technique, array-chips might be produced with nucleic acid strands to would be detected[110].

2.4.2.1.4 Microbial Biosensors

Microbial biosensors include microbial organisms such algea, bacteria, virus as bioelement in biosensor applications. Microbial biosensors are constructed by immobilization of microorganisms on transducer of biosensors. The changes in the vital activity of the microorganism like respiration, inhibition of some specific enzyme effected by the target analyte, on transducer surface are converted to electronic signal by electrochemical transducer[111]. This biosensors are employed in brewing, food manufacturing, waste-water treatment, energy production and pharmaceutical synthesis[3]. To increase sensitivity, selectivity and to develop versatile usage of microbial biosensors, genetically modified microorganisms are also used and studied[112].

Microorganisms have to be immobilized on transducer surface prorely to have effective biochemical response from transducer. Conventional microorganism immobilization techniques are adsorbtion, encapsulation, entrapment, covalent binding and cross linking methods[111]. Entrappment and encapsulation methods could be implemented together with more efficient results. S. H. Song et. al. measured bacterial denitrification

activity change as Ochrobactrum anthropi SY509 are immobilized by hybridization of entrapment technique and encapslation technique (Figure 2.12). Entrappment of bacteria was provided with gel formation of Poly Vinyl Alcohol (PVA) and boric acid. Encapsulation technique was implemented as forming a semipermeable membrane with Xanthan gum and Tween 20 which is an inert material. That semipermable membrane hold bacteria on transducer surface to get biochemical reaction effectively. In that study, it was discovered that hybridization of entrappment and encapsulation immobilization methods are 2.7 times more effective than just entrapment method[113].

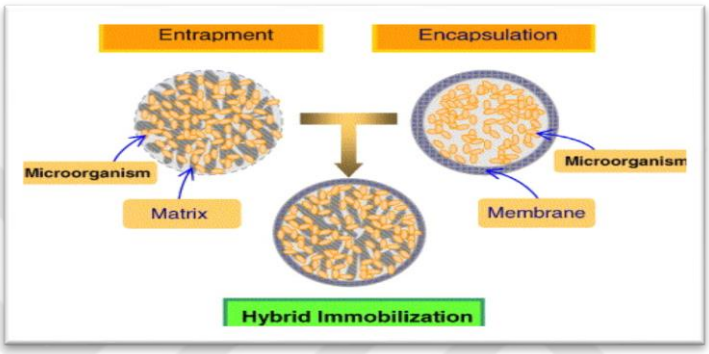

Figure 2.12Hybridization of entrapment and encapsulation methods for microorganism immobilization[113]

Ranjan et. al. observed whether there are heavy metals and pesticide in water by developing a biosensor with Photobacterium leiognathi. Photobacterium leiognathi is a luminescence bacreria which has lux gene and so luciferase enzyme. These bacterias emit light via lusiferase enzyme with lux gene. In that study, Photobacterium leiognathi was immobilized on transducer with appropriate matrix gel, and the medium of biolumenescence bacteria is prepeared by increasing luciferase enzyme production. Molecules like heavy metals and pesticide which cause biotoxicity reacts with some functional groups of biomolecules such as carboxyl, histidyl, thoil group. Hg(II), As(V), Cd(II) ve pestisit 2,4-dichlorophenoxyacetic acid inhibits lusiferase enzyme and so that light emmission of bacteria changes according to these toxic molecule concentrations. Lumnescence bacterias emit photon about 430 nm wave lenght in normal conditions but emition activity of photons change under stres condition via Hg(II), As(V), Cd(II) ve pestisit 2,4-dichlorophenoxyacetic acid and these situations are read from light emission spectroscopy[114].

In vitro, microbial biosensors are prefered to enzymatic bisensors because enzyme catalyzation is difficult in vitro application and bacterias are propagated easily in cell

culture medium to ready use. They can also tolerate more than enzymatic biosensors to small temperature and pH variations. In comparision with other cells (e.g. animal, plant cells), microbial cells are get more stabilize and remain alive for longer time in vitro. Microbial biosensors are typically cheaper than enzymatic biosensors without enzyme isolation procedure, with being less tolarable to inhibition because of other factors out of analyte. These advantages bring more advantage in fabrication of biosensors[115].

2.4.2.1.5 Carbohydrate Biosensors

Carbohydrate biosensors are fabricated with carboydrate bioelements. The main reason of the study on the carbohydrate biosensors is that there plenty biological reactions related with carbohydates. Carbohydrates make easier immobilization of bioelements on transducer and enhances biocampability[116]. Glucopolymers and synthetic saccharide derivatives are used as thin film layer on transducer surface and this provides to enhance sensitivity of biosensors[117]. Effectively immobilized carbohydrate on transducer enhances both productivity of reaction with analyte on transducer and signal amplification of biosensors. Dutra et. al. showed that linking a pneumocacal carboydrate to a specific protein A ocuured a chemical adsorbtion on silicon oxid wafer. This property amplifies signal of acustic biosensors[118]. Beak M. et. al. sythesized glucothiophene which is soluble in water and have mannoz, sialic acid ligands and these glucothiophenes are used as bioelement of biosensor to detect influenza virus and E. Coli. As soon as Influenza virus and E. Coli binds with sialic acid ligand and mannoz ligand of glucothiophene respectively, glucothiophene chains gives different absorbtion wave lenght by visibile absorbtion spectroscopy because of electroctatic and H bond conformational changes. As blue light is visible for only glucothiophene, red light is visible after interactions between Influenza virus A and sialic acid, E. Coli and mannoz on absorbtion spectroscopy. That study provides developing a carbohyrate biosensor to detect Influenza virus and E. Coli based on adsorbtion spectroscopy technique[119].

2.4.2.2 Classification of biosensors based on trancducer type

Transducer is the part of bisensors which converts physical, chemical and biological changes to readable signals. In other words, electrochemical interactions between analyte and bioelement on transducer are transmitted to electronic part of biosensor as readable signal via transducer of biosensor. Magnitudes of physical and chemical

changes could be small absolute scales such as nanogram, pikoamper, mikrovolt. Here it is that transducer part of biosensor is very important. There are various transducer types according to physical and chemical reactions in biosensors. Described transducer types in this study are electrochemical, optical, mass based (acustic), thermal and microcantilever biosensors. They convert these chemical or pysical changes which imply amount of analyte concentration to electrical signal for analysis. Under these titles, classification of biosensors have been elaborated according to transducer types used in fabrication.

2.4.2.2.1 Electrochemical biosensors

Electrochemistry has an important role in the research and development of biosensors.During the electrochemical reaction loss of electrons (oxidation) or gain of electrons(reduction) occur between electroliyte components. These reduction and oxidationreactions are known as redox reactions. After redox reactions, some prominent electrochemical analyzer provide informationabout theconcentration, kinetics, reaction mechanisms, chemical status, and other behaviorof a chemical species in electroliyte solution. Determination of electrocemical changes inbiosensing investigations is provided by relating with electrochemical reactions in nature and biological system. Electrochemical biosensors are designed with an electronic platform and bioelectrochemical component. Bioelectrochemical component/bioelement such enzyme is an element of data transmitter to transducer part of biosensor which converts signal to readable and quantifiable results[1]. In electrochemical biosensors, there has to be an electroactive bioelement or any intermediate produced from enzyme catalyzed chemical reaction. In other words, electrochemical interactions has to be occured for construction of any electrochemical biosensors. In electrochemical biosensor applications, various bioelements such as enzymes, microorganisms, proteins, oligonucleotides are used and especially enzymes are commonly used in development of electrochemical biosensors because of their specificity and bioanalitical capability abilities.Electrochemical biosensors are classified as amperometric, voltametric, potantiometric, conductometric and impedimetric biosensors[1], [120-122]. Electrochemical transducers are commonly used in biosensor applications because of the low cost, ease of use, fast analysis, portability, miniaturazation, small analyte volume and simplicity of fabrication[123-125].

In electrochemical biosensor applications, electrodes have an important role because electrochemical reactions generally occured on electrode surface or around electrode via immobilization of bioelements. Relating to interested function of electrochemical reaction; surface characterization of electrodes, including materials of electrodes substantially effects capacity of detection. Electrochemical sensing comprise of three electrodes which are RE, CE (auxiliary electrode) and WE (indicator, redox or sensing electrode). RE generally confects from Ag/AgCl materials and they have to be further removed from the redox reaction site to maintain a stable potantial. These electrodes have to be conductive and chemically stable. Typical WEs are platinium, gold, carbon materials are selective according to interested analyte type and a platinum wire is used coumter electrode[40], [81], [126]. In three electrode system, the charge from electrolysis passes through the counter electrode instead of the reference electrode because this protects the reference electrode from changing its half-cell potential. Electrochemical cell can consist only two electrodes without CE with the difference of three electrode system. These electrodes can be easily miniaturized at commonly microscale or nanoscale to have portable and design small biosensors. Microelectrodes, screen printed electrodes and interdigidated electrodes are functionalized by different designs of three electrode system[3].

Microelectrodes get easy to manifacture surface modification of electrode by deposition techniques such vapor deposition and allow integration microfluidic system on itselves to produce microfluidic chips.Screen printed electrodes (SPEs) have gained popularity in electrochemical biosensors due to high electrocatalitic activity, perfect compability, high sensitivity, stability, easy of use, low cost properties and ease and speed of mass production using thick film technology. SPE has provided ease of use fairly whereby working, reference and counter electrodes can be printed on the same substrate surface (Figure 2). This fabrication design also allows to develop portable and rapid electrochemical biochips for point of care SPEs can also be miniaturized and integrated microfluidic systems to produce microchips have versatile functions[53]. The patterned working electrode is typically made of conductive carbon and gold and used various electrochemical biosensor applications[6], [7], [127-130].

Nanomaterials as an important component in electrochemical biosensor applications are placed in recent years because of significant opportunities. Materials in nanoscale have opportunities in terms of sensitivity because there is a high compability with analyte

scale and nanomaterials increase surface area for bioelement immobilization so it allow more effective reaction on eletrode surface modified by nanomaterials[87], [131]. Some basic nanomaterials in electrochemical biosenosors are nanowires, nanorods, nanoparticles for development DNA biosensors, enzymatic biosensors and immunosensors[86], [132], [133]. Metalic nanomaterials turn a irreversible redox reaction on electrode into reversible as extremely decreasing potantial of some analitically significant electrochemical reactions. Metal oxide nanomaterials such Titanium (IV) oxide nanoparticles (TiO_2NP), Mangan(IV) oxide nanoparticles (MnO_2NP) are used in electrochemical biosensor application with large surface area, conductivity and biocompability.

2.4.2.2.1.1 Potantiometric biosensors

Potantiometry is the branch of electrochemical chemistry and potantiometric biosensor principle is that measuring accumulation of charge potantial between working electrode and reference electrode in electrochemical cell under the conditions of no current flow or negligible current flow. In other words potantiometry gives information about ion activity in electrochemical reactions by measuring difference of potantial between reference electrode and working electrode. The potantial differentiation in electrochemical cell occurs because of free energy change that would become if the chemical phenomena were to existed until the satisfied equilibrium condition. Potantial difference between electrodes is correlated with analyte concentration, so this principle is using development of potentiometric biosensors. After interaction between analyte and bioelement of biosensor there would be a change of electrons amount in electrochemical cell, potentiometry can provide to quantify the difference between the original potantial and the potantial after the interaction. Electrodes are commonly used in potantiometric biosensors can be pH sensible, univalent ion sensible glassy electrodes, anion/cation sensible ion-selective electrodes commonly for ions such as K^+, Ca^{2+}, Na^+, Cl^- and gas sensible (almost for CO_2 and NH_3) electrodes. Potantial difference in potantiometry can be measured between either an indicator or a reference electrode or two reference electrodes separated by a permselective membrane when the current flow is zero or negligible current[1], [120], [122], [134].

2.4.2.2.1.2 Amperometric biosensors

In amperometry, changes in current generated by the electrochemical oxidation or reduction in electrochemical cell are observed with time while a constant potential is maintained at the working electrode with respect to a reference electrode. Amperometric biosensor is an electrochemical biosensor which measure continuos current during oxidation or reduction of electroactive species occured in biochemical reaction at appropriate applied potantial value to the electrochemical cell[1], [81]. Amperometric biosensors can measure the current at constant potantial when flowing samples across the electrode as in flow injection analysis. Chronoamperometric measurement is applied in steady condition under experimental conditions avoiding convection as the mass transfer to the electrode. In chronoamperometry, mass transfer to the electrode is made by diffusion. Hydrodynamic amprerometric measurement is applied in movable solutions which are obtained by stirring solutions or moving working electrode in soutions. In both chronoamperometry and hydrodynamic amperometry, diffusion layer thickness on working electrode changes according to concentration gradient of electrolyzed substance which will be analyzed. In chronoamperometry, diffusion velocity and concentration gradient are directly proportional. In hydrodynamic amperometry, movement speed of solution is related to concentration gradient[135]. Density of current during electrochemical reaction is correlated with electroactive material concentration which is oxidised or reduced on working electrode in amperometric methods. After calibration of amperometric biosensor, current density is correlated with concentration of analyte[3].

Difference of amperometry from voltametry is the absence of a scanning potential. Difference from potentiometric biosensor is that output product of electrochemical reaction at working electrode is consuming. Amperometric detection is generally used in affinity and biocatalytic biosensors by reason of that has ease of use and low limit of detection. Amperometric assesments have additional favorable selectivity in that the oxidation or reduction potential used for detection is characteristic of the analyte species and amperometric electrodes have high biocatalytic activity and good time stability. During amperometric detection, the fixed potantial causes a negligible charging current which is needed to apply the potantial to the system. This stuation gets an advantage which minimizes the background signal that affects accurity of signal and the limit of detection negatively[3].

Enzyme based biosensors and immunosensors have been extensively developed with amperometric electrodes because of simple construction and use, and low detection limit. Other bioelements, are immobilized on amperometric electrodes, are cell, tissue and microorganisms[74]. Most popular amperometric biosensors are glucose biosensors which are also first developed biosensors[72].

To set an example among published studies, W. Tang et. al. developed a amperometric glucose biosensor based on he synergistic action of nanometer-sized TiO2 and polyaniline Figure 2.13 illustrates a typical chronoamperometric response of their biosensor to glucose followed by injection of glucose measured in phosphate buffer at 650 mV in reference to Ag/AgCl. The change in current response is proportional to glucose concentration as glucose is consumed at the glucose oxidase (GOD) modified Glassy Carbon electrode (GCE) surface[136].

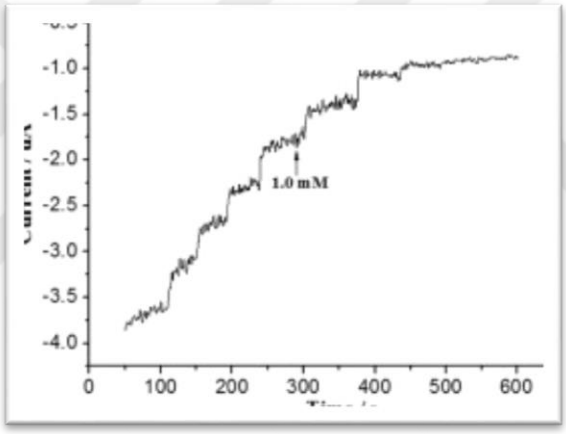

Figure 2.13Amperometric biosensor signal of the GOD/TiO_2/PANI/GCE-modified electrode in a stirred 0.1 M PBS solution (pH=7.0) for successive additions of 1.0 mM glucose at -0.45 V[136]

2.4.2.2.1.3 Voltametric biosensors

Voltametric biosensors measure the occured current and a sharp rise/fall of the current by applied increasing/decreasing potential to working electrode versus reference electrode in electrochemical cell until oxidation/reduction of the substance to be analyzed at the working electrode. This current is electrolysis current and is limited by mass transport rate of molecules to the electrode. There is a correlation between the concentration of the electroactive material and the hight of the peak current, this working principle creates the voltametric biosensor devices. In voltametry, three electrodes which are working, auxiliary and reference electrodes are employed to obtain

electrolysis current unlike potentiometry measurements which employ only two electrodes (working, reference). The use of three electodes system allows accurate application of potential functions and the measurement of the resultant current[3].

There have to be emphasised voltammetric analysis methods which are linear sweep voltammetry, hydrodynamic voltammetry, polarography, differential pulse voltammetry, square-wave voltammetry, ac voltammetry, stripping voltammetry and cyclic voltammetry. These methods obtain low level quantization with a wide dynamic range. Linear Sweep Voltammetry (LSV) is the one of the simplest voltametric analysis method by applying linearly varied potantial to a working electrode in time and at definite sweeping velocity. Occured current after the applied potantial imply a function of that potantial and is displayed as voltagram by computer connected electrochemical analyzer. The magnitude of the scan/sweep rate may be varied from as low as 1 mV/s to as high as 1000000 V/s. LSV are two types those are hydrodynamic voltammetry which buffer solution or electrode is continuously in motion and polarography which uses Mercury electrode as working electrode. In Differential Pulse Voltammetry (DPV), actuating signals are obtained by occuring periodic pulses during linearly scanning. Difference current (Δi) between at beginning and ending of applied pulses is recorded as a function of linearly increasing potantial. DPV can achive detection of concentrations as low as 10^{-7}-10^{-8} M. Square wave voltammetry is another voltammetric analysis method which current is measured at varied tiems by actuating pulse signal. Advantage of this method is having the fastest scanning among other voltammetry methods. Anodic Stripping Voltammetry (ASV) uses a mercury electrode as WE at a negative potantial to reduce metal ions in buffer solution and form amalgam with the electrode. The solution is stirred to carry analyte metal to the electrode as much as possible. Reduced and accumulated analyte on electrode for some time is reoxidized by the increased potantial on electrode and then this generates a current signal to corralate analyte concentration. ASV can achieve detection of concentrations as low as 10^{-10} M[3]. CV is commonly used and versatile electroanalytical analysis method to observe electroactive material behaviors. CV method description was done above in section 2.2.

2.4.2.2.1.4 Conductometric biosensors

Conductometric biosensors measure conductivity changes between reference nodes and electrodes. Conductivity changes occurs after interaction between analyte and

bioelement on conductor or semiconductor surface/active layer(polimeric thin film) or mediator(wire) between reference nodes and electrodes. Conductivity changes would be provided with enzymatic reaction whose charged products result in ionic strength changes, antibody-antigen interaction and DNA hybridization which obtain resistance on conductive surface substrate. Organic thin film transistors(OTFTs) works as conductometric biosensor principle. Organic thin film transistors-based biosensors (OTFTs) are the type of electrochemical transduced biosensors and classified into two types; organic field effect transistors (OFET) and organic electrochemical transistors (OECT) (Figure 2.14). A typical OFET is composed of an organic semiconductor film, a gate dielectric (insulator), and three electrodes (source, drain, and gate)[137]. Principle of device running is that source and drain with semiconductor are used to apply a source-drain voltage and measure the source-drain current that flows through the organic semiconductor, while gate is used to modulate the magnitude of the source-drain current. The gate can be used as a switcher of transistor. In OFETs the source-drain current is modulated by field eff ect doping, where the charge carrier density in the organic semiconductor is controlled by the gate via an electric field applied across dielectric layer[138]. Figure 2.14.a shows an OFET-based biosensor. In OECTs, the source-drain current is modulated by electrochemical doping or dedoping, where the change in the conductivity of semiconductor is caused by ions from electrolyte. Figure 2.14 b shows an OECT-based biosensor. Depending on the organic semiconducting material used in the active layer, the mobile charge carriers can either be electrons (n-type material) or holes (p-type material). So devices can be named n- type or p-type OTFTs according to the semiconductor charge carrier type[137].

In OTFT-based biosensor applications, sensing of any biological target molecule obtained whereby interaction between analytes and immobilized biological elements on organic active layer of biosensor occurs resistance on organic semiconductor layer. This resistance changes signals of current biosensor device acording to amount of attached target to biological elements on semiconductor layer. The signal change implies concentration of target molecules on semiconductor layer[138].

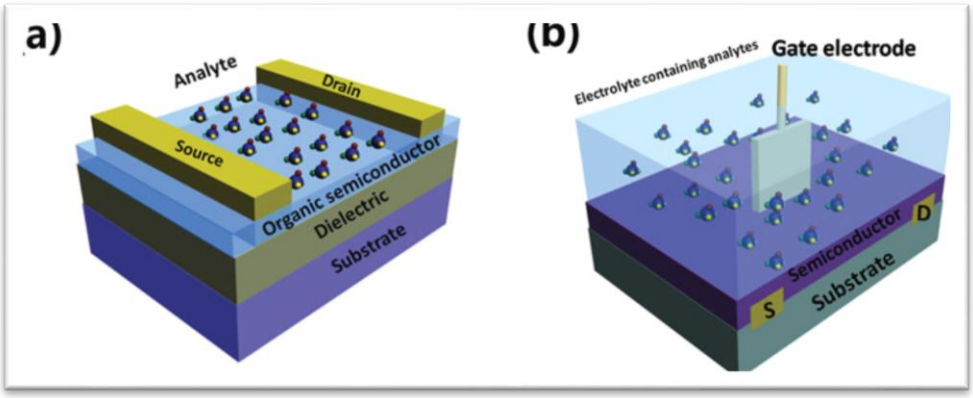

Figure 2.14a)A schematic of a typical OFET-based biosensor b) A schematic of a typical OECT-based biosensor[137]

2.4.2.2.1.5 Impedimetric biosensors

Impedimetric biosensors which is also known as capacitance biosensors measure capacitance change between electrodes in electrochemical cell with applied alternating voltage, amplitudes from a few to 100 mV are used. Impedimetric biosensors have recently gained popularity because of advantagous which are high sensitivity.Redox mediators have been used in the detection to be limited by the mediator's mass transfer rate in impedimetric biosensor applications. The Ab–Ag interaction helps to impedimetrically display to increase charge transfer resistance on the electrode layer. Ag concentration correlated with charge transfer resistance in impedimetric immunosensor. Using nanomaterials such as gold nanoparticles and carbon nanotubes in electrochemical impedance sensors is advantageous due to the increased electrode surface area, electrical conductivity on WE interface.

The electrode modification can be composed with various conductive materials such as polymers, metallic nonoparticles and small organic molecules by some techniques such self-assemble-monolayer(SAM), composite formation, electrochemical deposition and different adsorbtion methods (electrostatic adsorbtion, covalent binding attachment). After electrode modification with bioelement immobilization, the electron transfer resistance at the interface between the electrode and the solution changes slightly when analyte binds. This constructs the impedimetric biosensor working principle. In biosensor applications, impedimetric assesments are used primarily affinity biosensor which based DNA hybridization and immunological binding on electrode surface, where the slight changes in impedance are proportional to the concentration of interested analyte, the DNA single strand and antigen, respectively.

Impedance is expessed in equation 2.15; where Z is impedance, R is component resistance, C is capacitance.

$$Z^2 = R^2 + \frac{1}{(2fC)^2} \quad (2.15)$$

The expression for Z(ω) is composed of a real and an imaginary part. Plotting the real part on the horizontal axis and the imaginary part on the vertical axis results in the "Nyquist plot". In this plot, the vertical axis is negative and each point on the Nyquist plot is the impedance at one frequency. In the figure 2.15, low-frequency data are on the right side of the plot and higher frequencies are on the left.

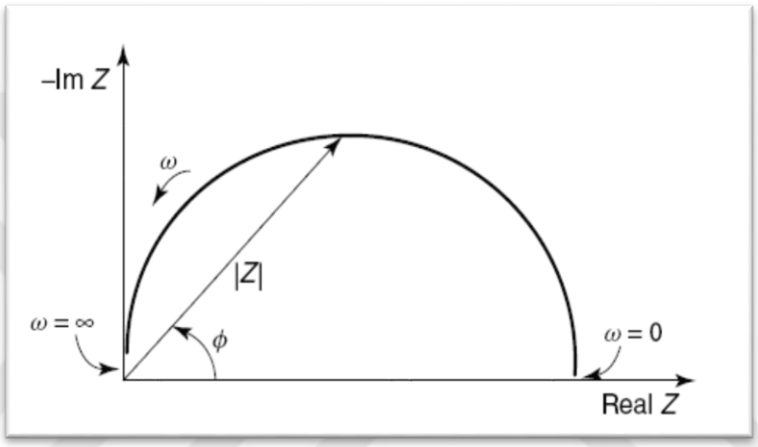

Figure 2.15Theoritically ideal Nyquist plotof Z(ω)

Electrochemical impedimetric biosensors are more convenient to development of label free detection. There are plenty of studies about electrochemical impedimetric spectroscopic biosensors. Rodriguez et. al. developed an aptamer (nucleic acid ligand) based label free impedimetric protein biosensor[139].

In some impedimetric immunosensor application, sensitivity of measurement can go down to femtomolar-fM (10^{-15})[15], but generally sensitivity of biosensors have a range of 1-10^{-9} M (M - pM).

2.4.2.2.2 Optical biosensors

Optic transducer based biosensors convert chemical or physicochemical changes because of analyte-bioelement interactions on optic transducers to quantifiable signals. Chemical and physicochemical changes on transducer are converted signals by light reflection, light polarization, light intensity, phase, peak position, and angular wavelength. Photon counter, florescence, luminescence, adsorbtion spectroscopies are

used as signal transducer in optical biosensor application. In this study, 8 types of optical biosensor are shortly introduced. Elipsometric biosensors, Surface Plasmon Resonance (SPR) biosensors, fiber obtic biosensors, absorbtion spectroscopy, interferometer based biosensors, RAMAN & FTIR can be improved for label-free detection. Florescence spectroscopy, luminescence spectroscopy generally employ by labeling analyte or bioelement with another materials such florescent dyes[140].

2.4.1.2.2.1 Ellipsometric biosensors

Ellipsometric biosensorsmeasure changes of light polarization or the polarization states after and before reflection after interaction between analyte and bioelement.Parts of elipsometric biosensors are polarizer, componsator, analyzer and detector. The incident light from the light source reflects and refracts further at each interface in the chip composed substances passing through a linear polarizer, a compensator in order and obtained ellipsometric data include information for investigated material within the penetration depth of the light, change of the polarization and refractive index values of a sample (Figure 2.16). In ellipsometric biosensor applications, sensormodeling is also needed because of their low refractive indexes and nanometer range thicknesses[141].

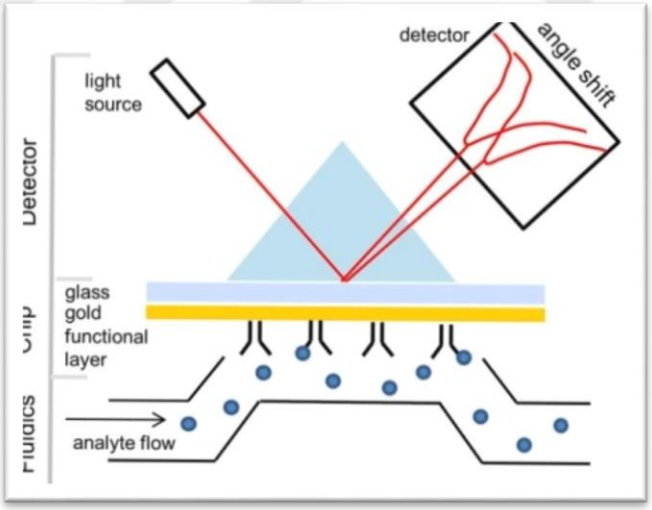

Figure 2.16 General schematic of an ellipsometric biosensor[141]

2.4.1.2.2.2 Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) biosensor was first demonstrated in 1983 by Liedberg et al.[142]. Measuring of the interaction between a wave vector induced by incident light beam and a wave vector in a metal film surface constructed by a dielectric material

coating on gold or silver generates basic principle of detection by SPR biosensor. Surface plasmon wave (SPW) is a electromagnetic wave that occurs at the interface of metal and dielectric media. SPW generates electromagnetic field at the interface of metal and dielectric media. There are four basic methods to set SPR principle which are prism coupling, waveguide coupling, fiber optic coupling, and grating coupling methods[140]. SPR via coupling prism method is configurated to clarify SPR principle in figure 13. In figure 13, a light wave passes through a high refractive index prism and is totally reflected at the interface and generates an evanescent wave at interface. This evanescent wave creates an evanescent field penatrated into the metal layer and excites molecules near the interface. This evanescent wave propagates along the interface with a propagation constant. The propagation constant of the SPW is sensitive to the refractive index of the dielectric. In SPR biosensor, after immobilized bioelements (antibodies) on the surface of metal recognize and capture analyte (antigen), a local increase in the refractive index at the metal surface occurs. Refractive index increasing at the metal surface generates an increase in the propagation constant of SPW propagating along the metal surface. The magnitude of SPW propagation constant change and refractive index change after bioelement-analyte interaction can be optically measured and related analyte concentration in SPR biosensor applications. By the other SPR structures, changes of some light characterists such as; light amplitude, polarization, spectral distribution can be correlated with the propagation constant of the SPW. Therefore, changes in the refractive index at the sensor surface after biosensing can be determined by change of SPW propagation constant via measurement of above mantioned light characteristics[140], [143].

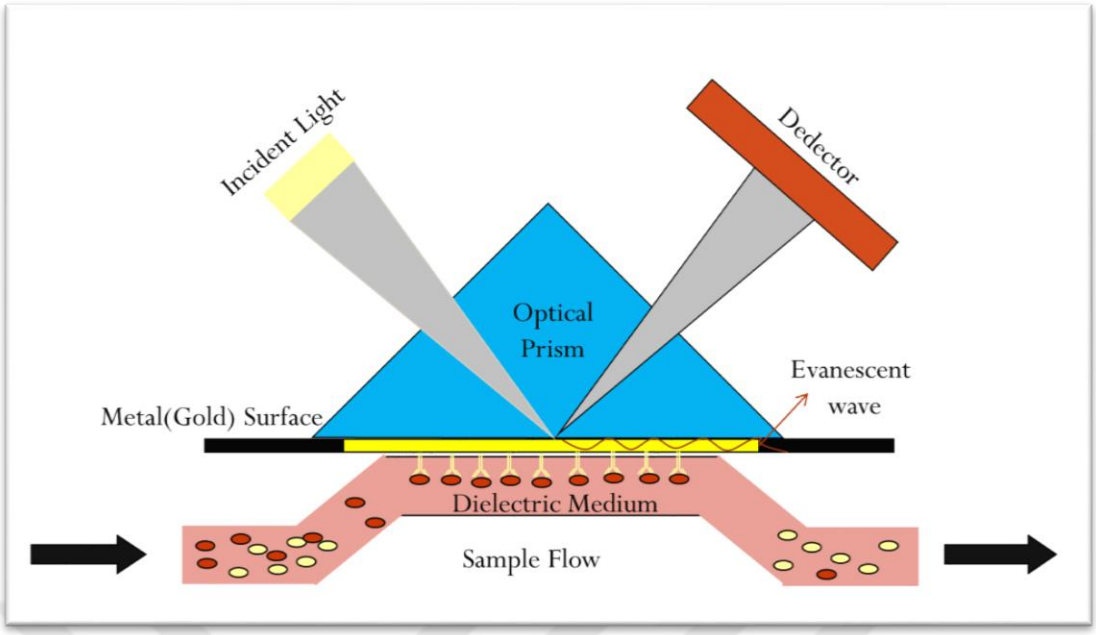

Figure 2.17 SPR biosensor via prism coupling configuration[143]

2.4.1.2.2.3 Fiber Obtic biosensors

Fiber-optic biosensors use optical fibers as transducer and generally include sensitive layer on the distal end of optic fibers. Light propagates by total internal reflection through fiber and this propagation consists of two components: the guided field in the core of fiber and the exponentially decreasing evanescent field in the cladding layer of fiber. Evanescent field decays to almost zero within the cladding layer in an uniform-diameter cladded fiber (Figure 2.18). Interactive relation between light and analyte-bioelement reaction can measured by adsorbance, luminescence spectroscopy, polar isolation and change of refrection index[144].

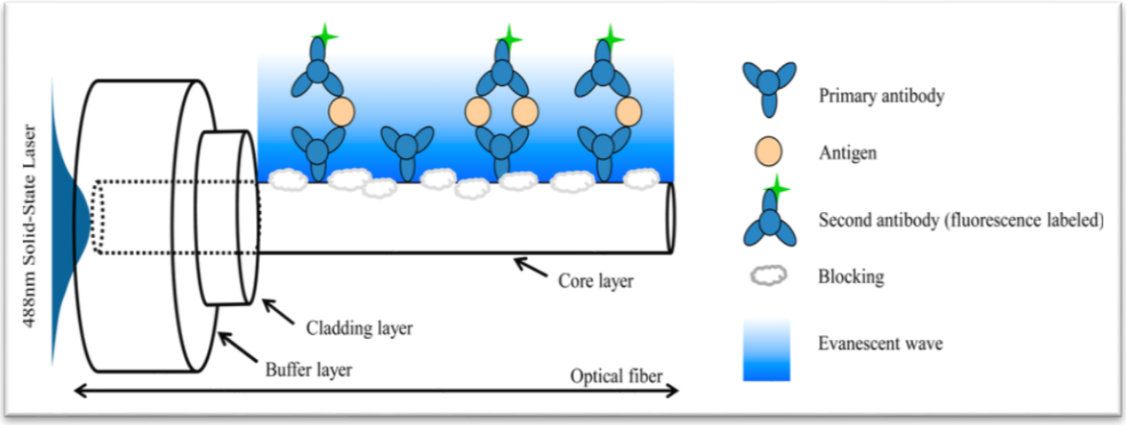

Figure 2.18 Fiber optic biosensor working principle based sandwich type immunosensor labeling by fluorescence tagged second antibody[145]

2.4.1.2.2.4 Absorbtion spectroscopy

Absorbtion spectroscopy measures change of absorbanse of light as transducer after interaction between anayte and bioelement. Absorbtion spectroscopy measures distinct energies after absorption of light by molecules which is produced with their transition from a ground state to excited state and then relaxation step. In absorbance based optic biosensors, the intensity of absorbance of light measured spectroscopy imply the presence of interaction between analyte and bioelement which can be correlated to analyte concentration[141].

2.4.1.2.2.5 Florescence spectroscopy

Florescence spectroscopy measures change of florescence light emmition of optic transducer surface bacause of interaction between anayte and bioelement. In floresence based optic biosensors, either analyte molecule or bioelement are labeled with florescent tags such as dyes; the intensity of florescence measured spectroscopy imply the presence of interaction between analyte and bioelement which can be correlated to analyte concentration[141].

2.4.1.2.2.6 Luminescence spectroscopy

Luminescence spectroscopy measures change of luminescence light emmition of optic transducer surface bacause of interaction between anayte and bioelement. In luminesence based optic biosensors, either analyte molecule or bioelement are labeled with luminescent tags such as dyes; the intensity of luminescence measured spectroscopy imply the presence of interaction between analyte and bioelement which can be correlated to analyte concentration[141].

2.4.1.2.2.7 Interferometer based biosensors

There are basicly three types of interferometer based biosensor designs which are Mach-Zehnder interferometer sensor, multi-channel Young's interferometer sensor and Hartman interferometer sensor[140]. The most popular design is Mach-Zehnder interferometer (MZI) biosensor among these designs which is firstly demonstrated by Heideman et al.[146]. In MZI, incident light beams from laser is splitted into two arms equally in a microfluidic system: reference arm which s protected from the sample with a thick cladding layer and a sensing arm which allow analyte-target interaction by

modification of microchannel. These two arms recombine at the output of chip, and a photodetector like charge-couple device camera measures the light intensity change resulting in interference. The presence of the analyte is determined by a phase difference relative to the reference because refractive index change on sensing arm (Figure 2.19)[140], [147].

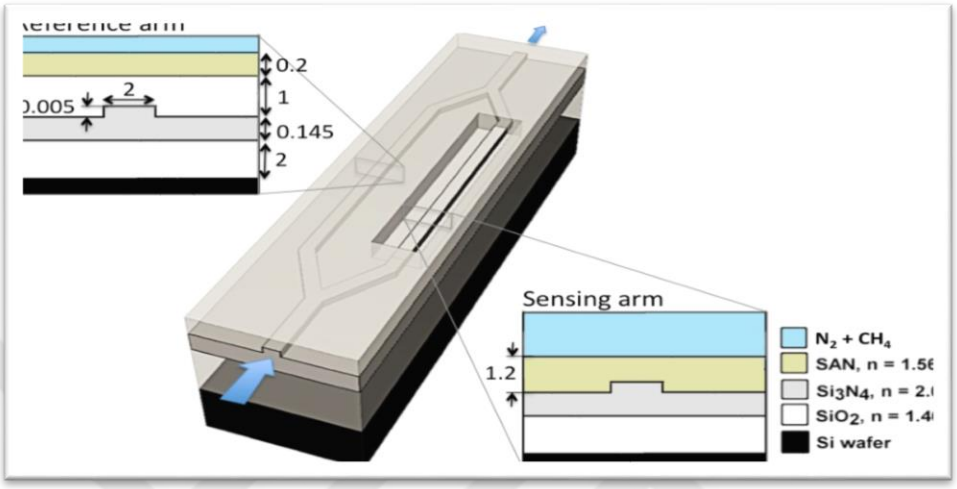

Figure 2.19Mach-Zehnder Interferometer configuration [148]

2.4.1.2.2.8 RAMAN & FTIR

A FTIR spectrometer equipped with a real time evacuation apparatus was applied to determine analyte concentration. A RAMAN spectrometer measures the vibrational energy spectrum depends on the chemical composition of the sample so that gives information about chemical specificity and represents a chemical fingerprint of the sample. These two spectrometers measure the interaction of energy with the molecular bonds in a sample of an unknown material.These spectrometers have being used in biological recognition in biosensor applications[149].

2.4.1.2.3 Acustic biosensors

Acoustic transducers measures mass changes on the biosensors surface. Piezoelectric crystals are the most common acoustic transducers which generate electric currents from a vibrating crystal because of absorbed mass on its surface after interaction between immobilized bioelement and analyte. Piezoelectric materials have ability of acustic waves production and propogation. All acoustic sensors are sensitive to changes in many physical or chemical parameters, like force, film thickness, mass, concentration, viscosity. Piezoelectric elements can be found in various electronic

devices, such as radar, computers, mobile phones, electronic blood presure monitor, and so on. Acustic biosensors easily allow improvement of label free detection because of responding to mass accumulation on the sensor surface. Another important advantage of acustic sensor is that can offer real-time measurement of surface interactions as an alternative to optical biosensors such as surface plasmon resonance and optical waveguides.

2.4.1.2.3.1 Piezoelectric

2.4.1.2.3.1.1 Surface Acustic Wave Sensors(SAW)

In 1880, Curie brothers first discovered piezoelectricity with the conversion between mechanical vibrations and electrical energy which occurs incrystals having one or more polar axes or in the absence of a center of symmetry.

The piezoelectric effect arises when pressure applied to a dielectric material deforms itscrystal lattice, which in turn causes a change in the distribution of charges in the atoms andbonds, generating a net macroscopic electrical polarization of the crystal.If electrical contacts (electrodes, electronic conducting films) are applied to the sides of athin slab or rod of a piezoelectric material, current will flow through an external circuitwhen stress is applied to the crystal. When the stress is released, then the current flows in theopposite direction. The typical acoustic device consists of a piezoelectric material with one or more metal transducers on its surface(s).

2.4.1.2.3.1.2 Quartz Crystal Microbalance(QCM)

Quartz Crystal Microbalance (QCM) is the oldestand the most commonly used as acousticsensor. QCM responce is related mass change on transducer after interaction between analyte and bioelement which would be detected[63].

2.4.1.2.3.2 Love Wave Senors

2.4.1.2.3.3 Magnetic Acustic Resonators

The acoustic element can be made from different materials, so the limitedconfiguration of traditional acoustic sensors can be improved considerably. Magnetic acustic resonators are over traditional acustic based transducers like piezoelectric materials.

Theadvantages of the magnetic acustic resonators are absence of direct electric contacts to the resonator, absence of pressure points, simplified fluidics, easy size reduction, choice of a variety of materials, integration into lab-on-a-chip technology, andmultifrequency operation[3].

2.4.1.2.4 Thermal biosensors

Thermal biosensors are also known as calorimetric biosensors which measure heat change after biolelement-analyte interaction on transducer surface. All chemical and biochemical processes involve the production or absorption of heat. This heat change is related to the amount of analyte to be detected. Termistors is generally used as signal transducer in thermal biosensor applications. In these type biosensors, the biggest challenge is statues of temperature increasing. So that, robust material for high temperature in exothermic reactions has to be used in fabrication of biosensors[150].

2.4.1.2.5 Microcantilevers

There are also thermal and micro cantilever based transducers are being used as detection devices which are based on a processes measuring the production or absorption of heat and the change in the resonant frequency of the cantilevers. A change on the cantilever surface after interaction bioelement and analyte in the resonant frequency of the cantilevers is caused by a change in mass and/or stiffness of the cantilever, and this change can be measured. Three general different modes are used in cantilever arrays as static mode, dynamic mode, and heat mode. Common to all modes is the fact that the cantilever's surface is usually functionalized in such a way that one surface is chemically active while the other surface is chemically passive as reference[151].

2.5 Cancer biomarkers and electrochemically immunodetection of cancer biomarkers

2.5.1 Cancer and Cancer Biomarkers

Cancer is a general disease name of uncontrollable and anormal cell division which is resulted by DNA damage on some particular gene. There are various types of cancer according to which related to gene. Cancer disease shows that very complicated molecular pathway is related to mutation of DNA sequence. In term of diagnostic of

cancer disease, molecular pathway analysis is significantly imply that some protein molecules are increasing or decreasing in progress of cancer. Clinical diagnostic of cancer with biosensor gets significant advantages those are; increasing flexibility and detection speed, multiple analyte detection cabability, ease of use with not sophisticated production process, economical advantages.

Biomarkers are molecules which are secreted after biological process in living organisms. Biomarkers help us to understand a disease at molecular level and contribute developing better drug for the diseases, defining new drug targets and determinining side effects of drugs as observing concentration in the body fluids like blood, urine, mucus, in tissue or in cell[152]. Biomarkers in cancer disease researches and diagnostic with different applications and which cancer type related are reviewed in table 2.2.

Table2.2 Cancer biomarkers and detection applications

Biomarkers	**Cancer Type**	**Detection Aplication**	**Reference**
ERBB2, IGFBP2	Synovial sarcoma	Microarray analysis	Ref[153]
Mitochondrial DNA mutation	Bladder, head, neck cancer	Nucleotide sequencing	Ref[154]
pGP9.5	Lung cancer	Gel-electrophoresis	Ref[155]
Mammaglobin	Breast cancer	Gel-electrophoresis	Ref[156]
Haptoglobin alpha subunit	Ovarian cancer	Protein electrophoresis	Ref[157]
CA-125, Osteopontin, Prostatin	Ovarian cancer	Gel-electrophoreis Immunoprecipitation cDNA microarray	Ref[158]
Galectin-3	Colon cancer	Protein electrophoresis	Ref[159]
Des-gamma carboxy-prothrombin	Liver cancer	Protein electrophoresis	Ref[160]
APC, CDH1, DAPK, TIMP-3, GSTP1, MGMT	Breast, esophogeal, cervical, colon, head and neck, kidney	Methylation analysis	Ref[161]
HER3	Breast	Immunohistochemical, Western Blot	Ref[162]

Cancer biomarkers diagnostic with electrochemical biosensors are being able to design a DNA based biosensor for detect damaged gene part because of cancer with DNA hybridization on transducer. DNA hybridization provides perfect selectivity.

2.5.1.2 CEA

Carcinoembryonic antigen (CEA) was discovered by Gold and Freedman in 1965 by isolation from adenocarcinoma. CEA molecule is in family of cell surface glycoproteins, a subfamily among Immunoglobulin Superfamily[41]and it is used as an important biomarker in human serum associated with colorectal, lung, breast cancer and ovarian carcinoma. Molecular weight of CEA is about 200 kDa[42]. CEA is composed by 641 aminoacids which are primarily included asparagin, glutamin, treonin and serin aminoacids. Major monosacharide part of the CEA molecule (2/3 of whole complex CEA molecule) is consist of N-acetil glucoseamine[43].

It is thought that CEA is metabolized in the liver however there isn't common view about the catabolizm of CEA molecule according to recent studies[44]. CEA is an oncofetal antigen which generates in the embryonic stage and is repressed in the postnatal stage. Mature human serum includes CEA at very low level. If there is high level of CEA in the mature human serum, it is recognized as malignite signs[45].

CEA molecule as a tumor marker is first defined in colon cancer and colorectal cancer specifically. However, it was determined that CEA level can be high in other cancer types and some pathological stuation out of cancer. In the cancer researches and treatments, CEA is the most frequently used tumor marker[44]. For instance, after the demonstration of CEA secration ability in the lung cancer process, it was investigated place of the CEA in lung cancer diagnosis, staging and prognosis estimation. In the process of non-small cell lung carcinoma, CEA is propounded as a benign tumor marker[44].

CEA can penetrate to body filuid due to being on cell surface. 97% of healthy and non-smoker people have less than 2,5 ng/ml CEA in serum. 19% of excessive smokers and 7% of former smokers have more than 5 ng/ml, and this is approved as a meaningful datain the cancer phenomena. Higher level than 20 ng/ml of CEA in serum indicates metastasis stage of cancer disease and cancer prognosticate of some cancer types such colon and pancreas[46].

2.5.2 Detection of cancer biomarkers by electrochemical immunosensors

Detection of cancer biomarkers which are protein structure and high level in human blood as a result of mutation on cancer related gene is an important and being developed

issue in the field of electrochemical biosensor applications. Detection of cancer biomarkers by electrochemical biosensors has the edge on conventional assesment methods like ELISA and radioimmunoassay. Advantages of electrochemical biosensors are being smaller, faster, cheaper devices, without radiation hazards, more sensitivity, allowing label free, concurrent detection, simple production, less time consuming with rapid detection, having longer shelf life, and not complicated procedure. Electrochemical biosensors with these priorities can prevail against sensitive detection of cancer diagnostic markers and this will substantially get easier early dianostic of cancer at beginning phases. Early diagnosis of cancer is crucial for the successful treatment of the disease and increase health standards of people. Owing to alowing electrochemical biosensors to miniaturization of required equipments for cancer biomarker assesments, economical advantages and portable developed devices are gained. Electrochemical biosensors are also developing for multiple detection of cancer biomarkers which provides single test for several cancer biomarkers instead of seperate tests for each biomarkers. Miniaturized chips for multiple detection provide taking less blood sample than conventional measurements methods, this gets easier hospital laboratuary procedures. As withstanding innovations in electrochemical biosensor applications are used to widespread clinical applications; like portability, low cost, ease of use and miniaturizable properties of electrochemial biosensors offer opprtunities for alternative-site testing (e.g. physician's office), emergency-room screening, bedside monitoring, or home self testing[58]. Representative examples of these capabilities are glucose test strips as disposable screen printed enzyme electrodes, hand-held battery-operated clinical analyzers for rapid point-of-care measurements of multiple electrolytes and metabolites and hand-held electrochemical devices for bedside blood gas monitoring[58].

First cancer marker detection by electrochemical immunosensor implemented by Aizawa et. al. in late 1970s. They developed an enzymatic immunosensor with a competitive assay of hCG (human chorionic gonadotropin) in connection by amperometric monitoring of enzymatic interaction between catalase and hCG[163]. There have been various developments in the field of electrochemical immunosensors to enhance more sensitive and accurate antigen detection. More frequent developments were made on amperometric, sandwich type amperometric, voltametric and impedimetric electrochemical immunodetection. The developments of electrochemical

immunosensors for cancer biomarkers detection was reviewed in Table 2.3 including immobilization of antibody by electrode modification in the articles published in last 10 years.

Toluidine blue is used for electrostatic adsorbtion in electrode modification procedure as aternative to Nafion film for detection of cancer biomarker because Nafion film suffers from the slow mass transfer through the film and partition of mediator[164].

Table 2.3 Electrochemical immunosensors developed to detect cancer biomarkers in last 10 years

Electrochemical Transducer Type	**Electrode Modification-Immobilization method**	**Bioelement (Antibody)/ Analyte (Antigen)**	**Detection Range**	**Limit of Detection (LOD) S/N or Standard Deviation**	**Labelling of bioelement/ Disposable**	**References**
Impedimetric	AuNPs/MWCNTs-CS-GCE Adsorbtion by SAM	Ab-CEA/CEA	0.3–2.5 and 2.5–20 $ngmL^{-1}$	0.01 $ngmL^{-1}$ (S/N = 3)	Label free/ Disposable	Ref[27]
Impedimetric	Au colloid/1,6 hexanedithiol-Gold	Ab-IgG/PSA	15.3-328.3 ngL^{-1}	4.1 ngL^{-1}	Au colloid Labeled Ab_2	Ref[11]
Impedimetric	Nonoporous gold film-GCE	Ab-PSA/PSA	0.05-26 $ngmL^{-1}$	3 $pgmL^{-1}$	Label free	Ref[17]
Impedimetric	PDICT/Cysteamine-Gold electrodeCross linking by DMF	MDM2 mAb-MDM2	1 $pgmL^{-1}$-1µgmL^{-1}	0.26 $pgmL^{-1}$	Label free	Ref[38]
Impedimetric	Thiourea-Gold electrodeSAM via covalent coupling	Ab-CEA/CEA	0.01–10 $ngmL^{-1}$	10 $pgmL^{-1}$	Label free	Ref[165]
Voltametric	CS-SPCE Cross linking by GA	mAb-AFP/mAb-CEA-AFP/CEA	5 $pgmL^{-1}$ -5 $ngmL^{-1}$	Respectively3.9 ve 3.5 $pgmL^{-1}$	AuNPs labeled Ab_2	Ref[7]

Table 2.3 (cont'd)

Amperometric	GR-NH_2 –SPCE Cross linking by GA	mAb-PSA/PSA	6 orders of magnitudes for analyte	0.46 $pgmL^{-1}$ (S/N=3)	HRP-Ab_2/AuNPs	Ref[14]
Amperometric	AuNPs/thionine/Nafion - Gold electrode Electrostatic adsorbtion	mAb-AFP/AFP	1–250 $ngmL^{-1}$	0.56 $ngmL^{-1}$ R.S.D = 6.5%	Label free	Ref[37]
Amperometric	AuNPs/ mixed 1,6-hexanedithiol/cysteamine-gold electrde SAMs	mAb-AFP/AFP	15–350 $ngmL^{-1}$	5 $ngmL^{-1}$	HRP labeled Ab_2	Ref[36]
Sandwich type Amperometric	AuNPs/nano chitosan/GCEBy covalent attachment	mAb-AFP/AFP	0.01–40 $ngmL^{-1}$	2.3 $pgmL^{-1}$	Au-Fe_3O_4/Ab_2 labeled antigen	Ref[4]
Sandwich type Amperometric	Protein A/AuNPs/thionine film-Gold By covalent attachment	mAb-CEA/CEA	0.01 to 200 $ngmL^{-1}$	1.5 $pgmL^{-1}$	HRP-nano GHS-mAb-CEA labeled antigen	Ref[166]

Impedimetric immunosensors are constructed with antibody immobilization of working electrode and their working principle is that ocuring a correlation between antigen concentration and obtained resistance after electrochemical Ab-Ag interaction. EIS is used to chracterize these type detections. Conductometric immunosensors measure conductivity change after Ab-Ag interaction on sensitive layer. Conductivity change on transducer surface is determined by ohmmeter or multimeter.

Amperometric immunosensor principle is based current change measurements at constant applied potantial. HRP[127], [167], alkaline phosphatase[168], laccase[169], cholinesterase[170], and glucose oxidase[171] are used for electroactive material production during Ab-Ag interaction as tagging agent for amperometric detection. Possible limitation of amperometric immunosensors is interference effects due to electroactive components, this can also prevent interpretation of current signal. Coating electrodes with different polymers has being studied to solve this issue[172]. Sandwich type amperometric assesments of cancer biomarkers are widely studied and developed; sandwich type assesment provide greatly amplified sensitivity by labeling second antibody with significant enzymes such HRP[166]. Labelling Ab_2 with such enzymes amplifies amperometric signals on the electrode and improve the sensitivity of immunoassay because these enzymes have a role of mediator to transfer the electron between hydrogen peroxide (H_2O_2) and electrolyte solution after electrochemical reaction between Ab_1-Ag-labeled Ab_2.

CHAPTER 3

METHODOLOGY

3.1 Materials and Devices

Screen Printed Gold Electrode (Dropsens, Spain), monoclonal anti-CEA, CEA, 1,6-hexadithiol, ethanol, sulfiric acid, hydrochloric acid, nitric acid, sodium citrate, albümin from bovine serum (BSA), phosphate buffer saline (PBS), Gold (III) Chloride solution, Potassium hexacyanoferrate (III), Potassium ferracynide trihydrate, Sodium borohydrate, potassium chloride (Sigma-Aldrich, USA), double distilled water and dry Nitrogen stream were used in experimental section of the study.

CV and EIS characterization was implemented by potansiostat/galvonastat (VersaSTAT 3 Princton Applied Research).CV are performed by potantial scanning between -0.76 – 0.8V with 100 mV/s scan rate andThe impedance spectra were recorded using a sunisoidal ac potantial perturbation of 5mV (rms) in frequency range 100000-0.05 Hz, superimposed on a dc potantial of +0.14V the potantial of the redox ferri/ferrocyanide couple by potansiostat/galvonastat device (Figure3.1).Simulation of electrochemical impedance data was obtained by Zsimpwin version 3.20[173].

Figure 3.1 Potansiostat/galvonastat device which is used in electrochemical experiments

SPGEs and a conducter cable which conducts screen printed electrode and VersaSTAT 3 potansiostat/galvonastat were purchased from Referans Kimya(Figure 3.2 and 3.3). Drop Sens SPGEs C220BT are specifically designed to work in solution by entirely immersing sensing area with gold working electrode, gold counter electrode, silver reference electrode and silver electric contacts. Diameter of working electrode is 1.6 mm(Figure 3.3).

Figure 3.2 Conducter cable for conductance of SPGE and potansiostat/galvonastat device

Figure 3.3 SPGE which is conducted by conducter cable

Morphological characterization and size interpretation of gold nanoparticle was applied with Field Emition Scanning Electron Microscopy (FESEM). Zeta potantial (Zetasizer/ Nano ZSP) and UV-vis spectroscopy was used to interpret AuNPs size.

In progress of thiol modification of SPGE working electrode with 1.6 mm diameter, a tool was designed to modify gold working electrode area (1.6 mm diameter)

Figure 3.4 Designed tool for thiol modification of gold working electrode area

3.2 SPGE Modification using gold nanoparticles and electrochemical characterization

3.2.1 Synthesis of colloidal AuNPs and Morphological characterization of AuNPs

In gold nanoparticles synthesis, Frens method[48] was used with little modification. Before synthesis, all glassware used for nanoparticle synthesis were throughly cleaned in freshly prepared aqua Regia which is composed 3:1-HCl:HNO_3 and then rinsed thoroughly doubly distilled water. 0.025g sodium citrate was added to 2.5 ml of water and this solution was quickly added to 100 ml boiling aquous solution containing 1ml of 1% (w/w) $HAuCl_4$ with vigorously stirring while heating and the mixture was kept boiling with 15 minutes. The heater was removed and the solution was cooled at room temperature naturally. The color of the AuNPs solution was claret. Colloidal gold solution was stored at 4 °C in brown glass bottle. Morphological characterization was interpreted with FESEM, Zeta potantial and UV-vis spectroscopy.

3.2.2 SPGE modification

First,prior to modification, SPGE was subjected electrochemical cleaning by applying 20 potantial cycles between -0.35-1.2V with gold working electrode, gold counter electrode and Ag reference electrode in 0.5M H_2SO_4. Electrochemical treatment was applied by potantially scanning with 100mV/s scan rate. After that, SPGE was washed with double distilled water and dried in a nitrogen stream at room temperature.

After SPGE cleaning, 10mM 1,6-Hexanedithiol (1,6-HDT) solution in ethanol was prepared to functionalize SPGE with thiol group by self assamble monolayer. 75µl 1,6-HDT solution dropped on working electrode by designed tool (Figure 3.4) to modify only gold working electrode surface for 20 hours at room temperature in dark. Thiolated electrode was first rinsed with ethanol then rinsed water and dried in a nitrogen stream.

15µl prepared collaidal gold solution was dropped on thiolated SPGE and incubated overnight at 4 °C. AuNPs modified SPGE was rinsed with water and 10mM PBS(pH=7.4) and dried in a nitrogen stream.

15µl of 30µg/ml of monoclonal anti-CEA in 10mM PBS was incubated for immobilization on AuNPs modified SPGE for 12 hours at 4 °C. After antibody incubation, SPGE was rinsed with 10mM PBS then dried with nitrogen stream.

Blocking reagent was prepared with %0.5 BSA solution to block nonspecific binding side of monoclonal CEA antibodies. 15µl of BSA solution was added monoclonal anti-CEA adsorbed antibody dor 1 hour at 37 °C in incubator. After blocking of nonspecific binding sides of antibodies, 15µl of CEA antigen solution with 3 different concentration in 10mM PBS(pH=7.4) was dropped on working electrode of SPGE for 1 hour at 37 °C in incubator.

3.2.3 Electrochemical Characterization

Electrochemical characterization was performed after each surface modification and after each different CEA concentrations. All cyclic voltammetry experiments was performed in 5mM $K_4[Fe(CN)_6]$ and 5mM $K_3[Fe(CN)_6]$ redox couple in 0.1 M KCl, 20mM PBS at room temperature. CV are performed by potantial scanning between -0.76 – 0.8V with 100 mV/s scan rate.

3.2.4 Electrochemical Impedance Spectroscopy (EIS)

In this thesis, transducer part of the biosensor were studied because Au working electrode were being used as conductor in transducer part of biosensor. Modification of Screen Printed Gold Electrode(SPGE) allows an electrochemical change on transducer of our biosensor and this changes are converted to quantifiable signals by Electrochemical Impedance Spectroscopy (EIS) and Cyclic Votagram(CV).

The impedance spectra were recorded using a sunisoidal ac potantial perturbation of 5mV (rms) in frequency range 100000-0.05 Hz, superimposed on a dc potantial of +0.14V the potantial of the redox ferri/ferrocyanide couple.All electrochemical impedance spectroscopy experiments was performed in 5mM $K_4[Fe(CN)_6]$ and 5mM $K_3[Fe(CN)_6]$ redox couple in 0.1 M KCl, 20mM PBS at room temperature.Simulation of electrochemical impedance data was obtained by Zsimpwin version 3.20[173].

CHAPTER 4

RESULTS AND DISCUSSION

After gold nanoparticle synthesis, morphological analysis was performed with FESEM (Figure 4.1). UV-vis spectroscopy (Figure 4.2) was applied and Zeta potantial of colloidal gold solution (Figure 4.3)gave information about gold nanoparticles sizes. Synthesised AuNPs by Frens method produces AuNPs with reducing gold ions in $HAuCl_4$ by citrate molecules. The reduced gold atoms in solution, aggregate and form clusters, these clusters grow by increasing the number of atoms until achive the particles form. In AuNPs synthesis, trisodium citrate acted as a capping agent and thus restricted the growth of Au[47]. UV-vis spectrum gave about 520 nm and FESEM images implied that sythesized colloidal gold nanoparticles are about 40-45 nm.

On the gold electrode surface, the self assable monolayer of chemisorbed 1,6 hexanedithiol has beed used as the base interface for the deposition of synthesised Au colloid by Frens method[48] onto Au surface. Thiol group of 1,6 hexanedithiol strongly binds gold surface due to the soft character of both Au and thiol[49]. Other thiol group of 1,6 hexanedithiol was free to bind with Au colloids introduced in solution. After surface modifications, monoclonal anti-CEAs were adsorbed on AuNPs deposited gold surface. Electrochemical characterization methods performed after each modification and anti-CEA/CEA interaction. These electrochemical characterization methods were cyclic voltametry and electrochemical impedance spectroscopy.

CEA antigen proteins were incubated for an effective immmunochemical interaction on anti-CEA/BSA/AuNPs/1,6-HDT modified SPGE for 1 hour at 37 °C and pH 7.4 in incubator. After immunochemical interactions with 3 different concentrations of CEA proteins were detected by correlation difference resistance of R_{CT}-R_{SOL} and concentration changes of CEA in 10 mM PBS.

4.1 AuNPs morpological analysis

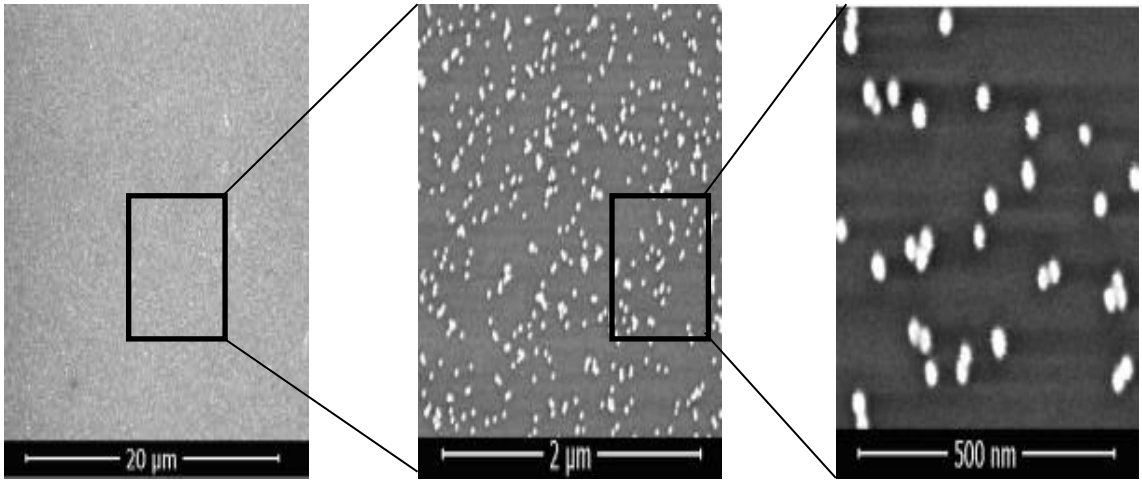

Figure 4.1 FESEM image of AuNPs in 3 different scales

The diameter of the AuNPs was measured by UV-Vis measurements and field emision scanning electron microscope. UV-Vis spectroscopy in Figure4.2yielded an absorbance maximum at 520 nm wavelenght and FESEM images in Figure 4.1 showed that the avarage size of the AuNPs was about 40-45 nm.

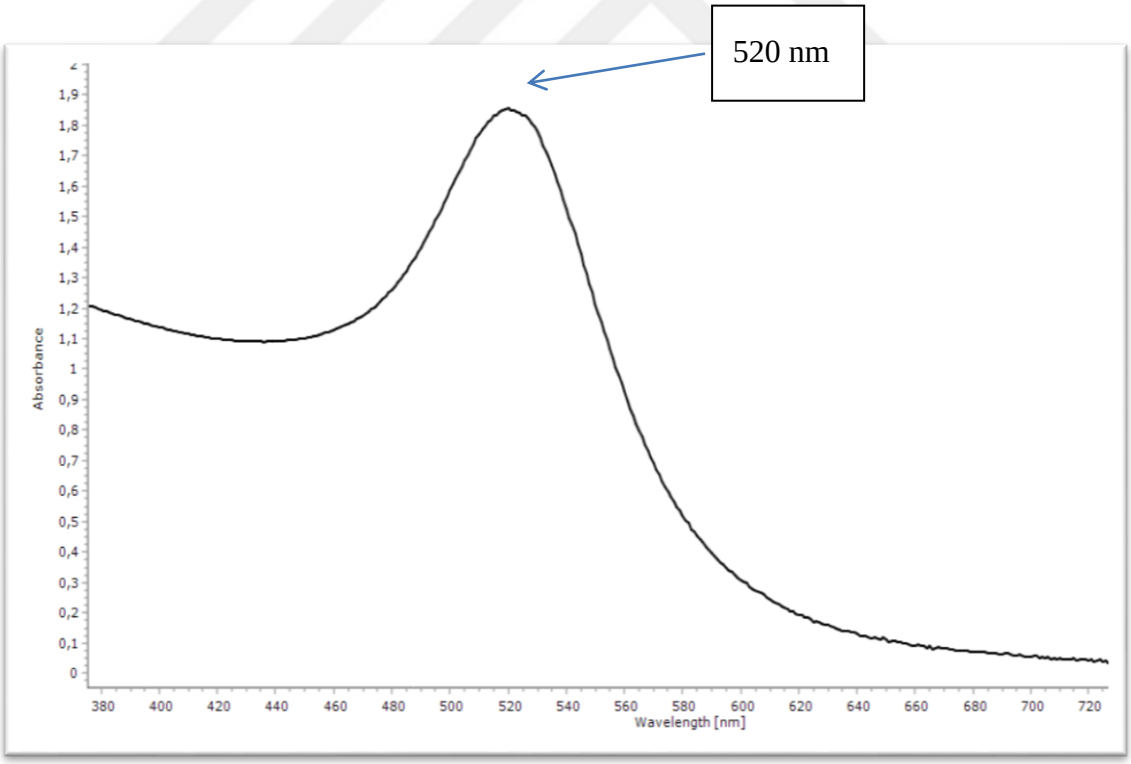

Figure 4.2Uv-Vis spectra Au colloidal solutions

AuNPs capped with trisodium citrate in collaidal solution carry an electririal charge so the zeta potential which is the electrostatic potential generated by the accumulation of

ions at the surface of the colloidal particles is used for electroactive AuNPs diameter interpretation. Zeta potantial analysis implied that avarage diameter of AuNPs is 46,30 nm. In conclution, as interpreting these used three characterization method implied that diameter of synthesised AuNPs by Frens method is about 40-45 nm.

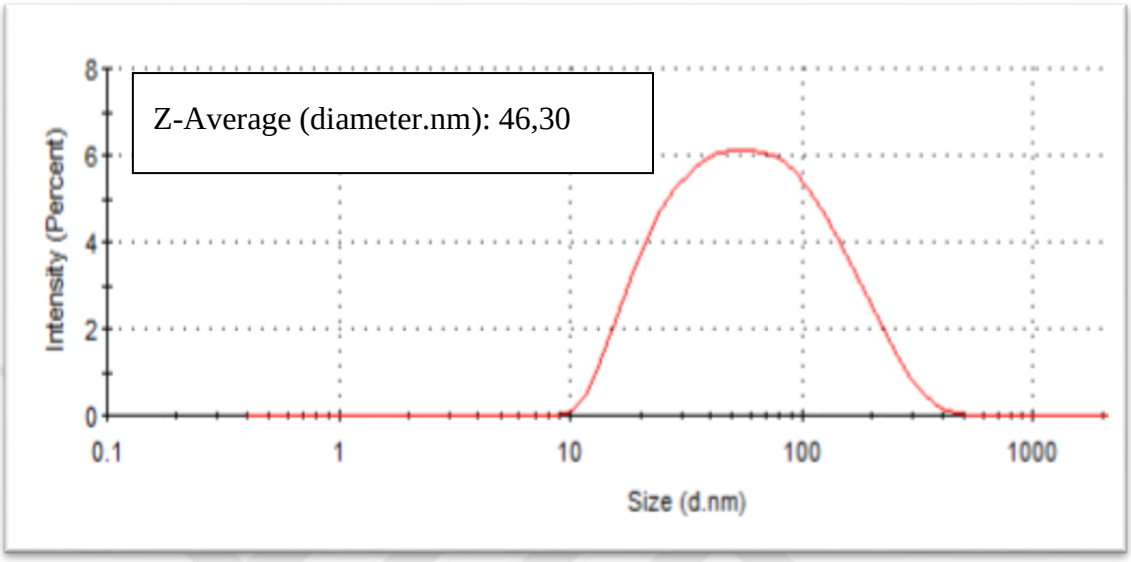

Figure 4.3 Zeta potantial measurementof AuNPs

4.2 Electrochemical analysis

4.2.1 Cyclic Voltammetry

Electrochemically cleaning of SPGE by applying 10 potantial cycles between -0.35-1.2V with in 0.5M H_2SO_4 were shown in figure 4.4. CV of electrochemically cleaning achived reproducable cycles at the end of cleaning. The purpose of the using H_2SO_4 is that deoxygenation comparision between other gold surface cleaning methods for electrochemical detection applications is very powerful and this method has easy procedure[174].

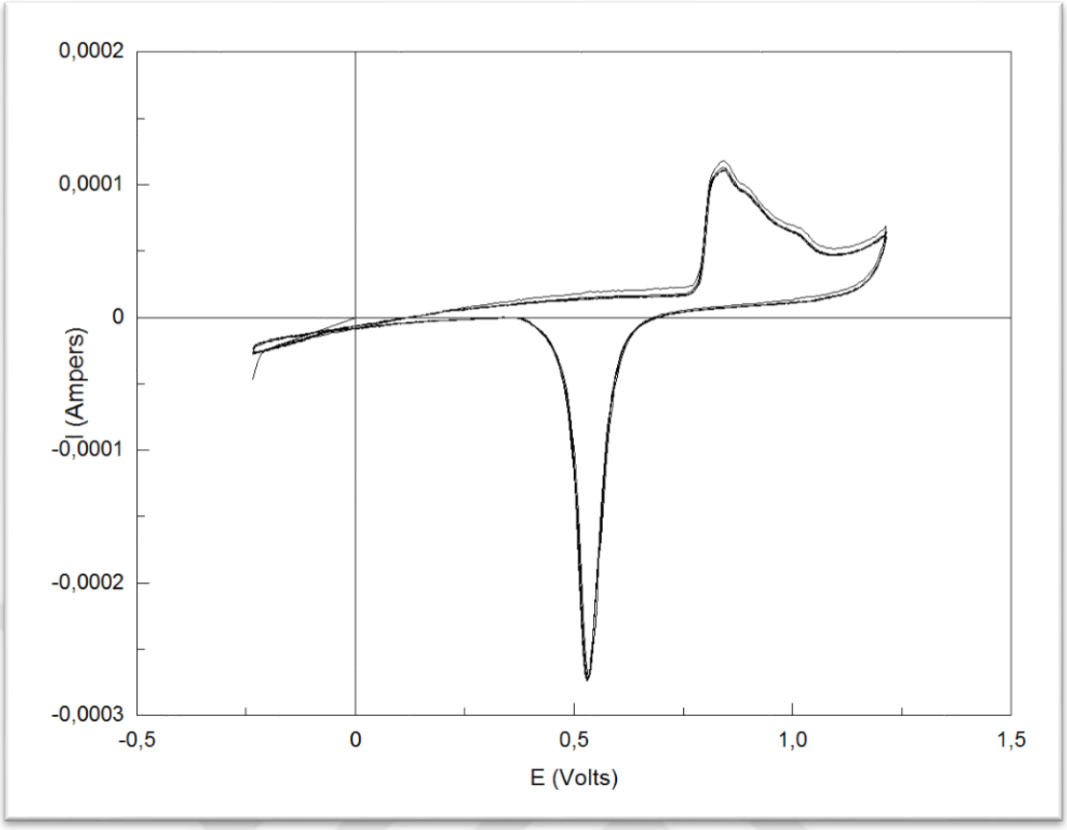

Figure 4.4 Electrochemically cleaning of SPGE in 0.5M H_2SO_4 solution by potantial scanning between -0.35 to 1.2 V with 100 mV/s scan rate until repeated cycle (10 cycle)

All electrochemical measurements were performed in5mM $K_4[Fe(CN)_6]$ and 5mM $K_3[Fe(CN)_6]$ redox couple in 0.1M KCl, 20 mM PBS.The CV of ferricyanide was chosen as a marker to investigatethe changes of electrode behaviour after each surface modification to develop for CEA detection on gold working electrode. The potential at the gold working electrode was controlled vs silver reference electrode. The controlling potential that was applied across the WE and the counter electrode was the excitation signal. The excitation signal was varied linearly with time; first scan positively. Then the potential was scanned in reverse, causing a negative scan back to the original potential to complete the cycle. CV were performed by potantial scanning between -0.76 – 0.8V with 100 mV/s scan rate and this scanning providedCV plots (Figure 4.5, 4.6, 4.7)of the response current at the working electrode to the applied excitation potential.

Anodic electrode process as the potential was scanned positively and was sufficiently positive to oxidize $Fe(CN)_6^{4-}$ was implied in equation 4.1.

$$Fe(CN)_6^{4-} \rightarrow Fe(CN)_6^{3-} + e^- \quad (4.1)$$

The electrode acts as an oxidant and the oxidation current increases to a peak. The concentration of $Fe(CN)_6^{4-}$ at the electrode surface depleted and then the current decayed. As the scan direction is switched to negative, for the reverse scan the potential is still sufficiently positive to oxidize $Fe(CN)_6^{4-}$, so anodic current continues even though the potential is now scanning in the negative direction. When the electrode became a sufficiently strong reductant, $Fe(CN)_6^{3-}$, was reduced by the cathodic electrode processas the potential was scanned negatively and was sufficiently negative to reduce $Fe(CN)_6^{3-}$ was implied in equation 4.2.

$$Fe(CN)_6^{3-} + e^- \rightarrow Fe(CN)_6^{4-} \quad (4.2)$$

In the positively scanning, $Fe(CN)_6^{3-}$ was electrochemically generated from $Fe(CN)_6^{4-}$ in the anodic process and in the negatively scanning, this $Fe(CN)_6^{3-}$ was reduced back to $Fe(CN)_6^{4-}$ in the cathodic process.

CV peaks are effected by magnitude of current, concentration of electroactive substances, transfered electron quanification, surface area of working electrode and diffusion coefficient. Thus, stability of redox reactions byproducts and possible another reactions which effect redox reaction can be analyzed[51], [56]. In biosensor applications, CV is used to analyze redox reaction kinetic and behaviour of electroactive substances in electrolyte solution in which an electrochemical interaction occurs between analyte (Ag) and immobilized bioelement (Ab) on WE. By CV method, whether electrode modification is successful and in parallel with this, whether modified electrode has conductivity and allows electroactive materials accumulation are informed. In this CV analysis, $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ redox probes were performed electron transferring on electrode with high speed, reversibly. CV characterization by redox probe is fulfilled as comparing bare electrode voltamogram and modified electrode voltamograms[56-58].After each gold WE modification step CV parameters were recorded as CV diagram in Figure 4.5.

After electrochemical cleaning in 0.5M H_2SO_4, SPGE was modified with 1,6-HDT. 1,6-HDT self assamble monolayer decreased electroactivity of bare electrode. As it obvious from Figure 4.5 the CVs of $[Fe(CN)_6]^{3-/4-}$ redox couple system at the bare SPGE (curve a), thiolated gold working electrode of SPGE (curve b) and AuNPs deposited on

thiolated SPGE (curve c) showed a reversible one electrode redox wave, the peak current of which being considerably increased after the modification SPGE with AuNPs. In fact, the AuNPs modifiedon thiolated SPGE was provided a large effective surface area of increased conductivity, as it compared with thiolated surface. To increase surface area and to provide antibody adsorbtion gold nanoparticles were deposited on thiolated SPGE. When 1,6-hexanedithiol was adsorbed at the surface of gold working electrode to form AuNPs deposition, the corresponding CV (curve b), showing a decreases in current response and an increase in the peak-to-peak separation between the cathodic and anodic waves of the redox probe, indicated that the electron transfer of $[Fe(CN)_6]^{3-/4-}$ was blocked. AuNPs were returned electroactivity back. And AuNPs increased electroactivitity compared to bare gold electrode with a little difference (curve c). It indicated that the AuNPs could enhance the efficiencyand the rate of electron transfer at the SPGE surface(Figure 4.5).

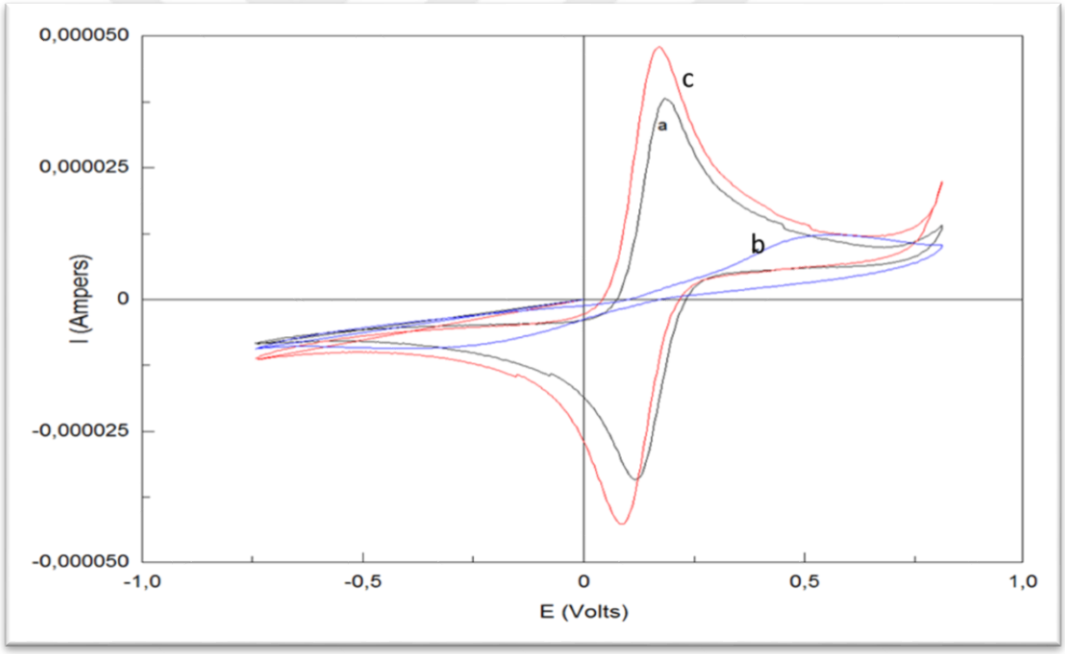

Figure 4.5 CVs of modified SPGE, curve a is bare electrode, curve b is thiol modified gold electrode, curve c is AuNPs modified electrodeby potantial scanning between -0.76 – 0.8Vat a scan rate of the 100 mV/s

In figures4.6, 4.7 and 4.8 implied that bare electrode, thiol modified SPGE and AuNPs adsorbed SPGE had reproducable CV signals. This was related stability of the modified gold electrode which was checked by a repetitive potantial sweep at a scan rate of the 100 mV/s and the resulting CVs (10 cycles) were presented in figures 4.6, 4.7 and 4.8.

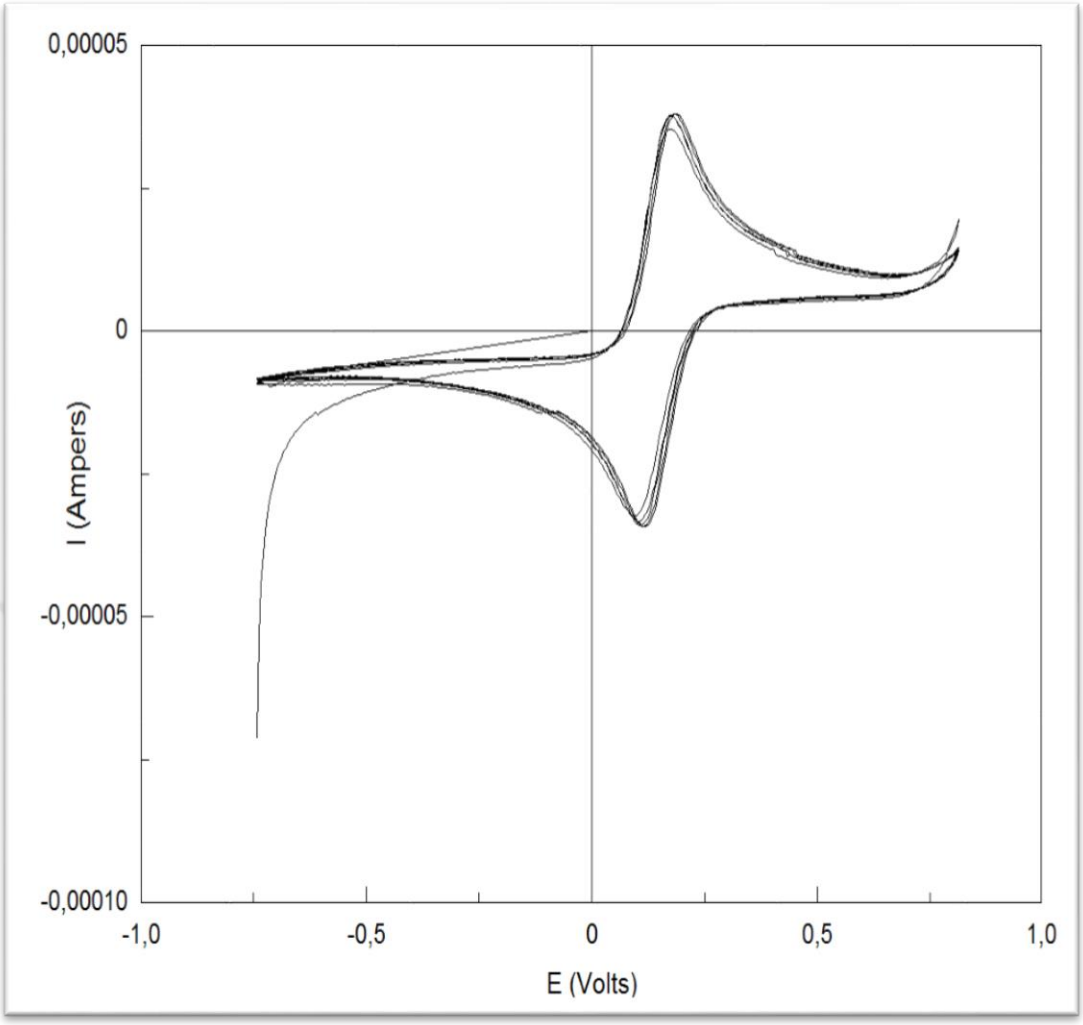

Figure 4.6 CVs of bare electrode in 5 mM redox probe by potantial scanning between -0.76 – 0.8Vat a scan rate of the 100 mV/s

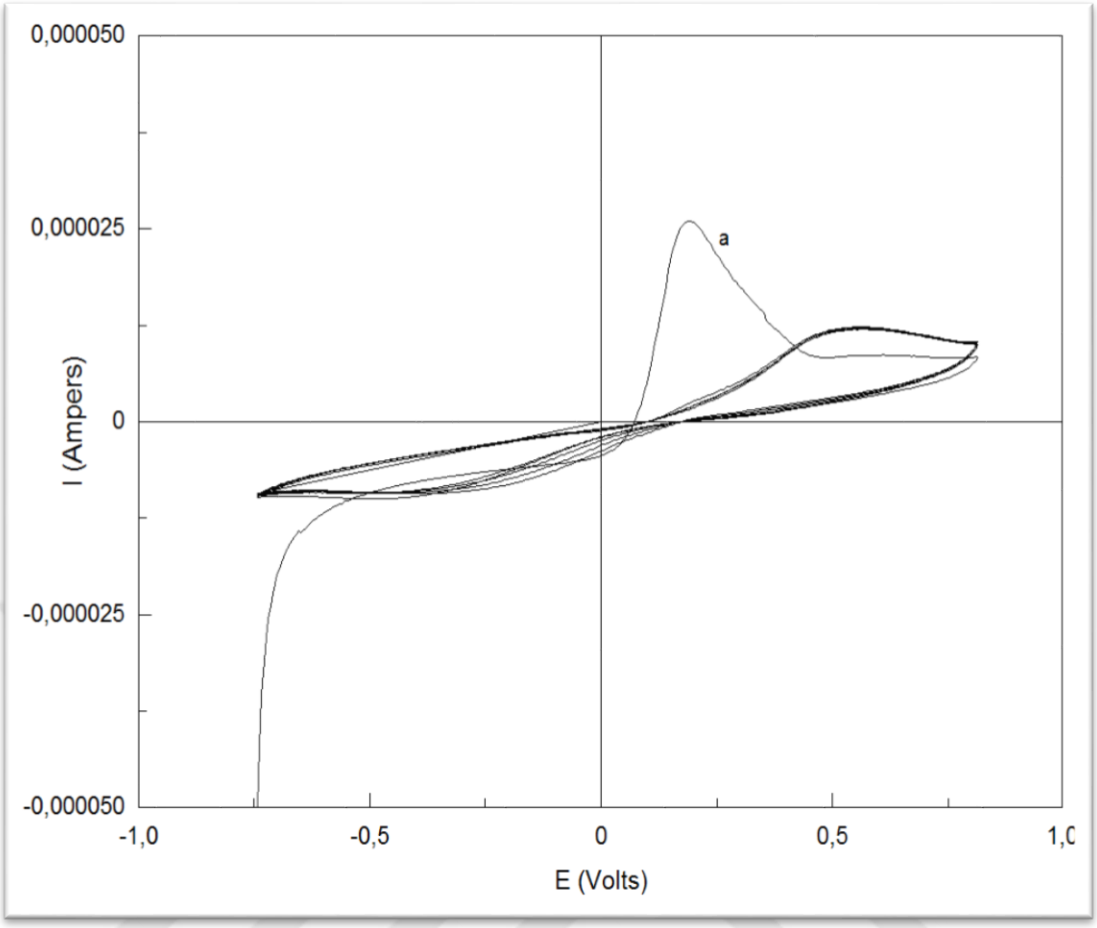

Figure 4.7 CVs of Au-thiol electrode in 5 mM redox probe by potantial scanning between -0.76 – 0.8Vat a scan rate of the 100 mV/s

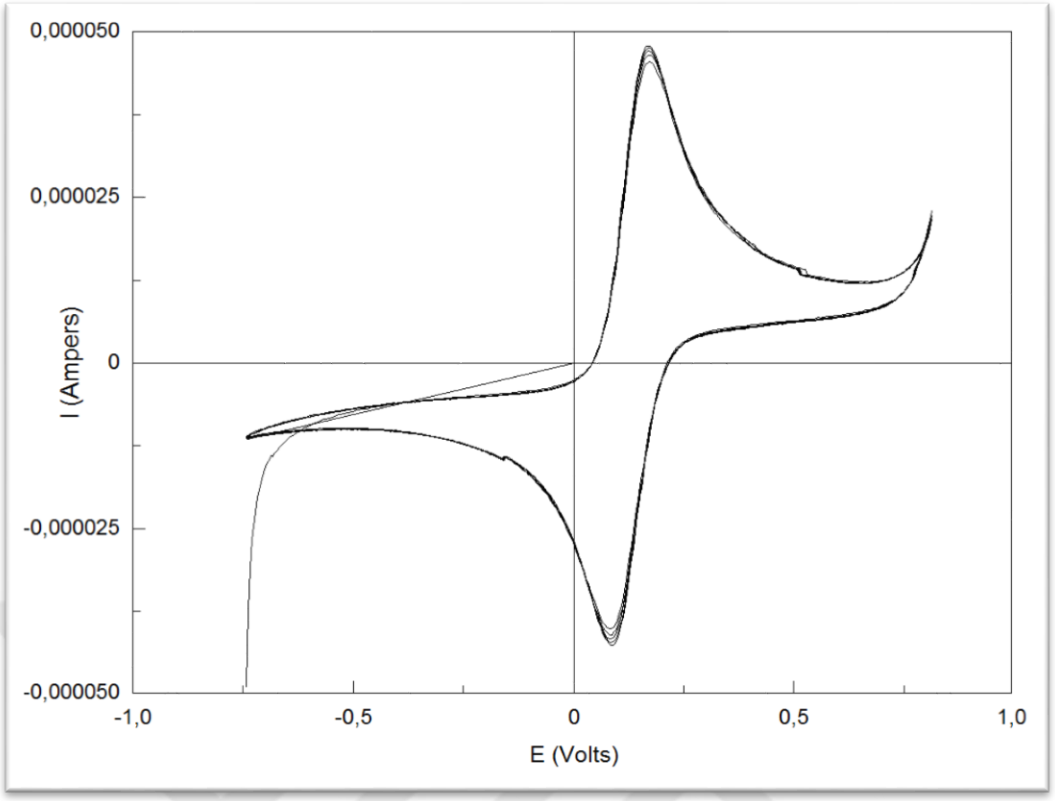

Figure 4.8 CVs of Au-thiol-AuNPs electrode in 5 mM redox probe by potantial scanning between -0.76 – 0.8V at a scan rate of the 100 mV/s

As expected, immobilization of monoclonal anti-CEA on AuNPs modified SPGE, once decreased the current response of the probe, due to the resulting decrease in the conductivity of the SPGE (Figure 4.9-curve a). Blocking active site of monoclonal antibody with BSA built up insulating layer on Au electrode and hindered the interfacial electron transfer. Finally, the interaction of CEA with monoclonal anti-CEA which was blocked active sites by BSA decreased the current of the redox couple (Figure 4.9-curve b) because the complex between anti-CEA and CEA on the surface of the SPGE acted as a nearly inert electron transfer blocking layer and hindered the diffusion of the redox couple to the surface of the SPGE.

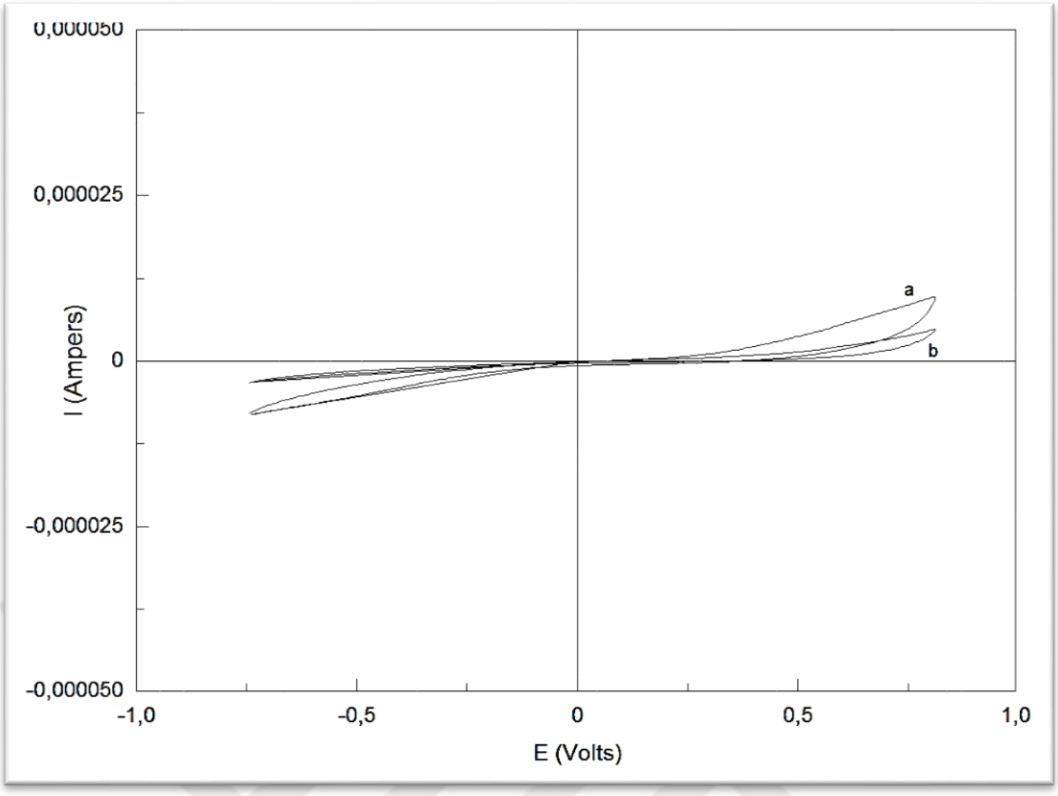

Figure 4.9 CVs of monoclonal anti-CEA immobilization on the modified SPGE, curve a is anti-CEA/BSA immobilization, curve b is anti-CEA/CEA interaction on modified electrodeby potantial scanning between -0.76 – 0.8Vat a scan rate of the 100 mV/s

4.2.2 Electrochemical Impedance Spectroscopy

EIS measures impedance (Z) by applying a sinusoidal (ac) voltage across an electrochemical cell and measuring the resulting ac current along with phase shifts between the ac voltage and ac current. In EIS, a sequence of impedance measurements include a semicircle portion starting at an initial frequency which has higher frequencies corresponding to the electron transfer limited process and a linear part at lower frequency range representing a diffusiion limitted process. The DC potential within the EIS action can be applied as a step for a constant voltage relative to reference for the entire EIS experiment. The semicircle diameter of the Nyquist plot in the impedance spectrum is a measure of charge transfer resistance, R_{CT} which controls the charge transfer kinetics of the redox probe at the electrode interface and obtains suitable signal for characterization of the modification at each step. Therefore, R_{CT} was correlated with different concentration of CEA molecule adsorbtion on modified SPGE.

In figure 4.10, Randless modified model was used as an equivalent circuit to fit all data of electrochemical mesurement parameters, where is R_{SOL}, solution resistance; R_{CT},

electron transfer resistance; C_{DL}, double-layer capacitance; W, Warburg diffusion impedance. The model which is presented in figure 4.10 involves Warburg impedance to consider the diffusion controlled process at low frequency region. Electrochemical data analysis for all EIS was performed using Zsimpwin version 3.20[173].

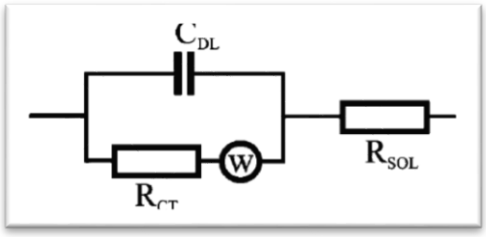

Figure 4.10 Equivalent circuit mold for complex impedance plots. R_{SOL}, solution resistance; R_{CT}, electron transfer resistance; C_{DL}, double-layer capacitance; W, Warburg diffusion impedance due to diffusion of the redox couple ($[Fe(CN)_6]^{4-}$ / $[Fe(CN)_6]^{3-}$) to the interface from the bulk of the electrode

The impedance spectra were recorded using a sunisoidal ac potantial perturbation of 5mV (rms) in frequency range 100000-0.05 Hz, superimposed on a dc potantial of +0.14V the potantial of the redox ferri/ferrocyanide couple.All electrochemical impedance spectroscopy experiments was performed in 5mM $K_4[Fe(CN)_6]$ and 5mM $K_3[Fe(CN)_6]$ redox couple in 0.1 M KCl, 20mM PBS at room temperature.

As it is obvious from Figure 4.11 the electrochemically cleaned bare SPGE revealed the smallest semicircle domain comparing other EIS measurements, because there were no surface modification on gold surface. Figure 4.12 implied that charge transfer resistance increased after AuNPs adsorbtion on thiolated by 1,6 hexanedithiol gold surface with diffusion increasing compared with anti-CEA/BSA adsorbtion. Anti-CEA/BSA adsorbtion had more stability on the interface, implying lower warburg effect. Anti-CEA/BSA adsorbtion had higher $\Delta R_{(CT-SOL)}$ than AuNPs adsorbtion which was corresponded diameter of Nyquist plot semicircle implying higher charge transfer resistance of the redox probe. This is because anti-CEA/BSA is served as an insulating layer and, consequently, decreased the interfacial charge transfer between the electrode and the electrolyte solution.

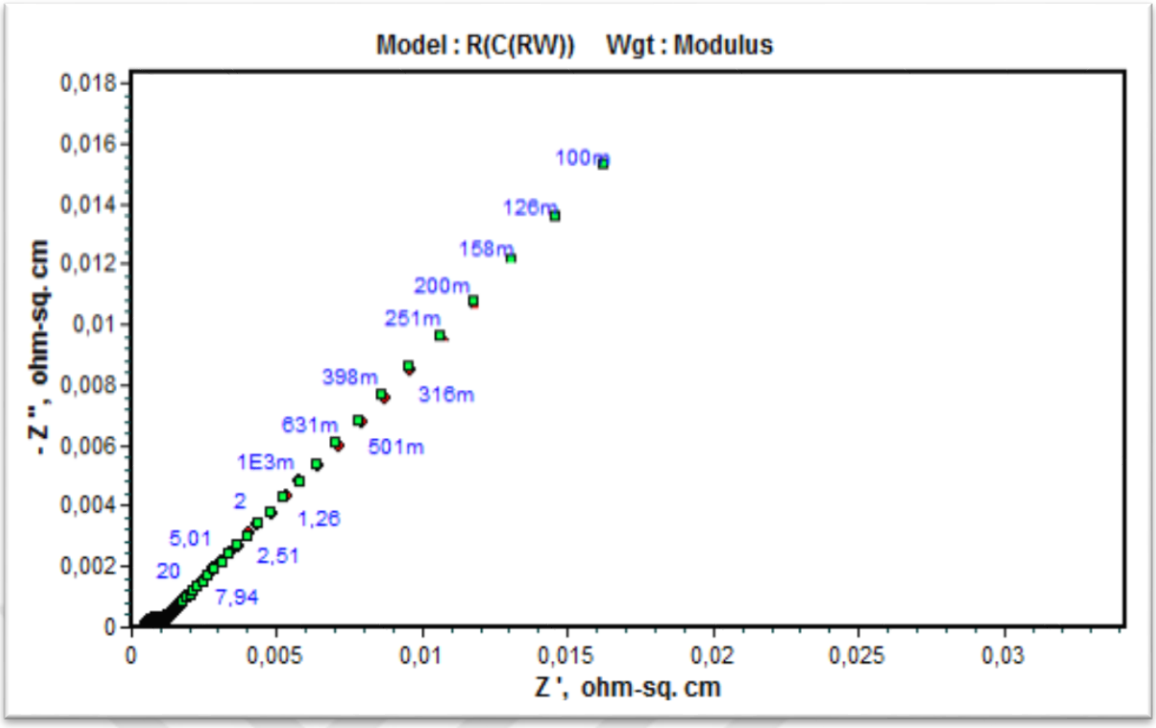

Figure 4.11 Nyquist diagram of EIS for electrochemically cleanedbare SPGE. The impedance spectra were recorded using a sunisoidal ac potantial perturbation of 5mV (rms) in frequency range 100000-0.05 Hz, superimposed on a dc potantial of +0.14V the potantial of the redox couple($[Fe(CN)_6]^{4-}$ / $[Fe(CN)_6]^{3-}$)

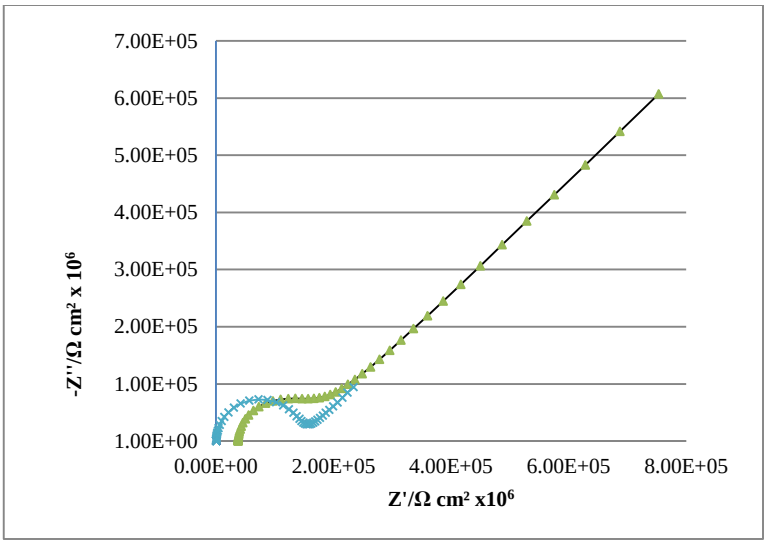

Figure 4.12 Nyquist diagram of electrochemical impedance spectroscopy for SPGE surface modification to develop an immunosensor. Green line is 1,6-hexanedithiol/AuNPs modified SPGE, blue line is monoclonal antibody with BSA adsorbed on 1,6-hexanedithiol/AuNPs modified SPGE. The impedance spectra were recorded using a sunisoidal ac potantial perturbation of 5mV (rms) in frequency range 100000-0.05 Hz, superimposed on a dc potantial of +0.14V the potantial of the redox couple ($[Fe(CN)_6]^{4-}$ / $[Fe(CN)_6]^{3-}$)

After immobilization of monoclonal antibodies CEA/BSA through adsorbtion on AuNPs, the interfacial charge transfer was inhibited, which lead to an increase in charge transfer resistance of the system (Figure 4.13). This was due to the generation of an insulating anti-CEA/BSA protein layer at the surface of AuNPs, which resulted in a higher charge transfer resistance and enlarged diameter of semicircle in the Nyquist diagram presented in figure 4.13.

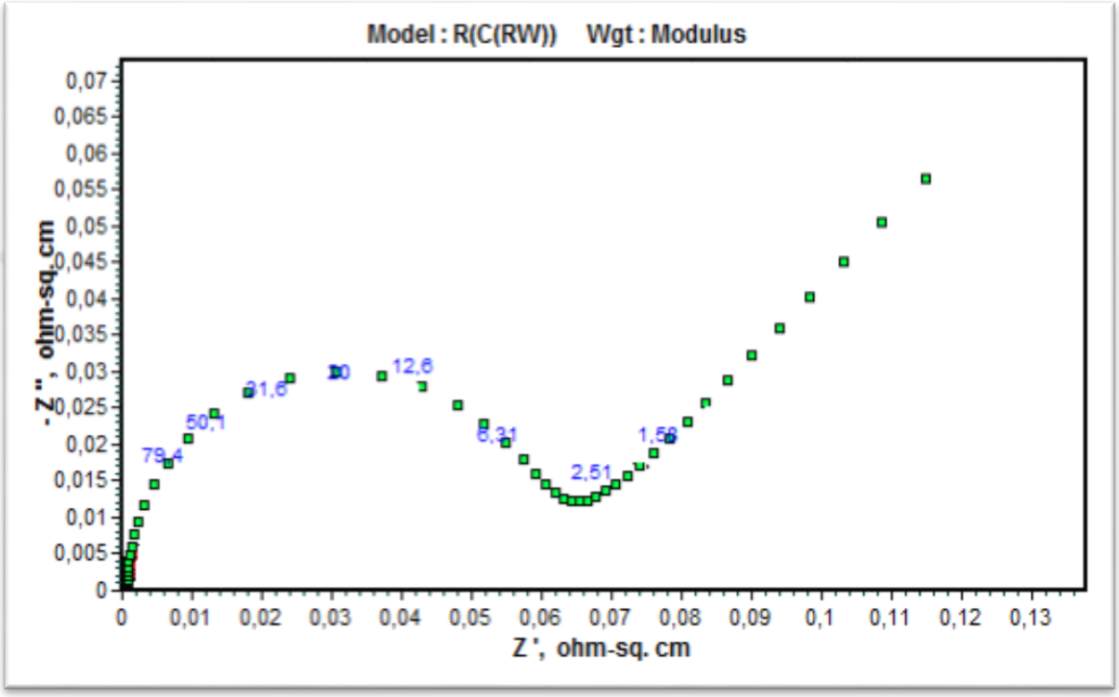

Figure 4.13 Nyquist diagram of electrochemical impedance spectroscopy for 1,6-hexanedithiol/AuNPs/anti-CEA/BSA modified SPGE in 10mM PBS. The impedance spectra were recorded using a sunisoidal ac potantial perturbation of 5mV (rms) in frequency range 100000-0.05 Hz, superimposed on a dc potantial of +0.14V the potantial of the redox couple($[Fe(CN)_6]^{4-}$ / $[Fe(CN)_6]^{3-}$)

EIS is a reliable and sensitive technique for small concentration. CV of the Ab-Ag interaction implied that electroactivity was incredably decrased after Ab-Ag electrochemical interaction (Figure 4.9). So that, electrochemical impedance spectroscopic anaysis of concentration had importance to detect Ag. Impedance change was correlated with detected CEA concentration change (Figure 4.14, 4.15 and 4.16). In Electrochemical Impedance Spectroscopy, semicircle diameter of EIS Nyquist plots implies electron transfer resistance, R_{CT}.The interaction of CEA with the monoclonal antibody CEA modified SPGE further increased the R_{CT}, as the formation of the corresponding adduct as a charge transfer blocking layer at the surface of the anti-

CEA/BSA/AuNPs/1,6-HDT/SPGE can largely hindered the diffusion of the redox couple to the surface of the SPGE.

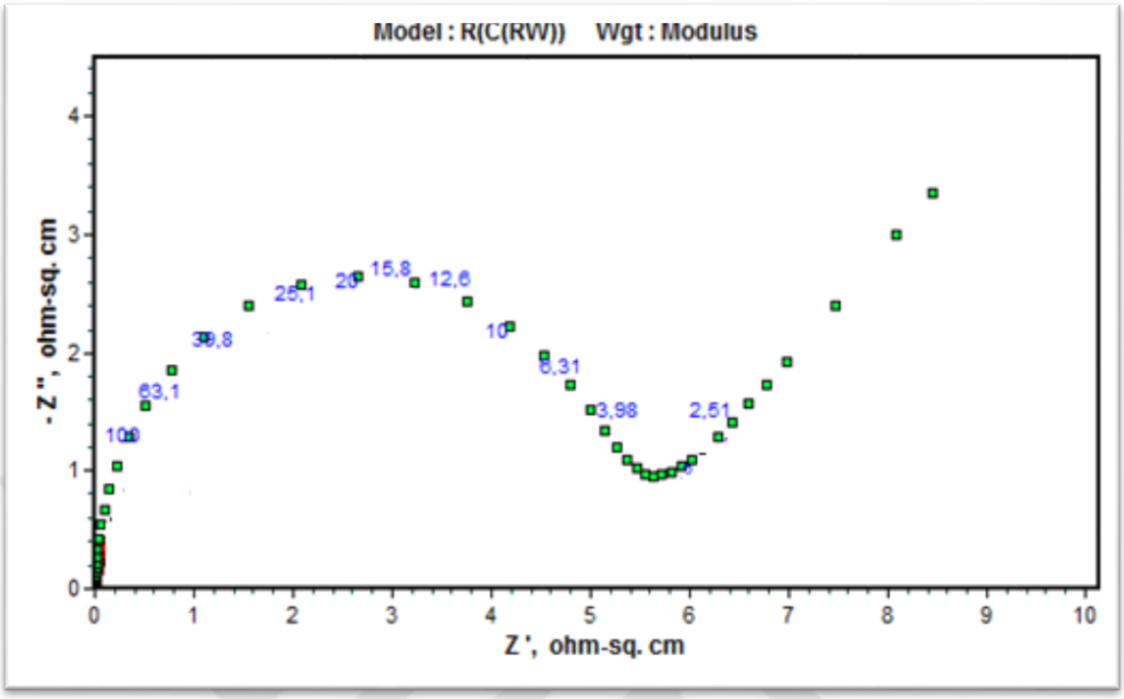

Figure 4.14 Nyquist diagram of electrochemical impedance spectroscopy for 1,6-hexanedithiol/AuNPs/anti-CEA/BSA modified SPGE with 25ng/ml CEA protein concentrationin 10mM PBS. The impedance spectra were recorded using a sunisoidal ac potantial perturbation of 5mV (rms) in frequency range 100000-0.05 Hz, superimposed on a dc potantial of +0.14V the potantial of the redox couple($[Fe(CN)_6]^{4-}$ / $[Fe(CN)_6]^{3-}$)

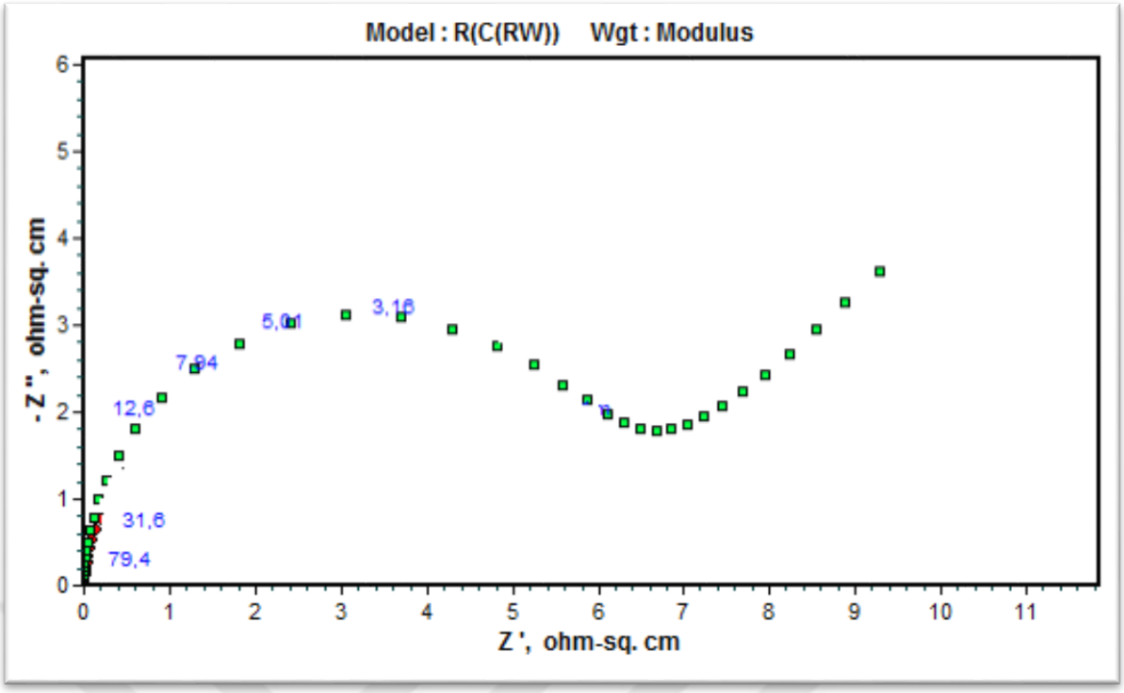

Figure 4.15 Nyquist diagram of electrochemical impedance spectroscopy for 1,6-hexanedithiol/AuNPs/anti-CEA/BSA modified SPGE with 50ng/mlCEA protein concentration in 10mM PBS. The impedance spectra were recorded using a sunisoidal ac potantial perturbation of 5mV (rms) in frequency range 100000-0.05 Hz, superimposed on a dc potantial of +0.14V the potantial of the redox couple($[Fe(CN)_6]^{4-}$ / $[Fe(CN)_6]^{3-}$)

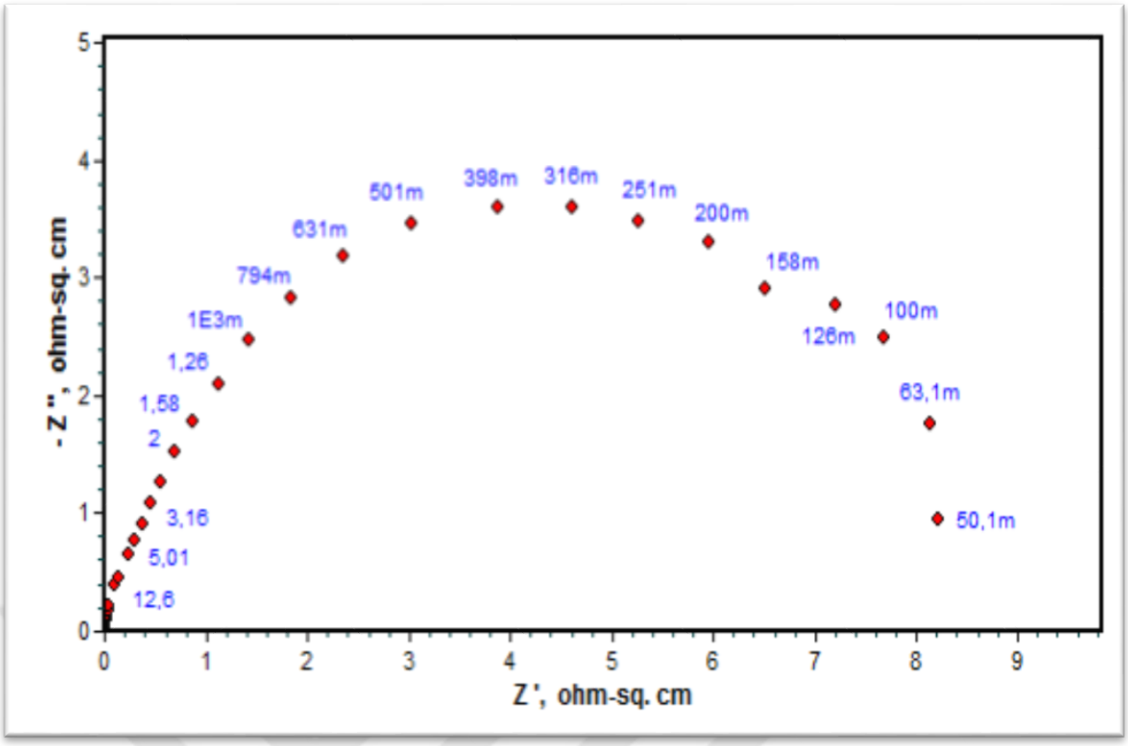

Figure 4.16 Nyquist diagram of electrochemical impedance spectroscopy for 1,6-hexanedithiol/AuNPs/anti-CEA/BSA modified SPGE with 100ng/mlCEA protein concentrationin 10mM PBS. The impedance spectra were recorded using a sunisoidal ac potantial perturbation of 5mV (rms) in frequency range 100000-0.05 Hz, superimposed on a dc potantial of +0.14V the potantial of the redox couple($[Fe(CN)_6]^{4-}$ / $[Fe(CN)_6]^{3-}$)

CEA antigen proteins were incubated for an effective immmunochemical interaction on anti-CEA/BSA/AuNPs/1,6-HDT modified SPGE for 1 hour at 37 °C and pH 7.4 in incubator. After immunochemical interactions with 3 different concentrations of CEA proteins were detected by correlation difference resistance of R_{CT}-R_{SOL} and concentration changes of CEA in 10 mM PBS. As it is seen from Nyquist plots presented in figures 4.14, 4.15 and 4.16, the R_{CT}-R_{SOL}increase along with the increasing CEA concentrations which are 25 ng/ml, 50 ng/ml and 100 ng/ml, respectively.

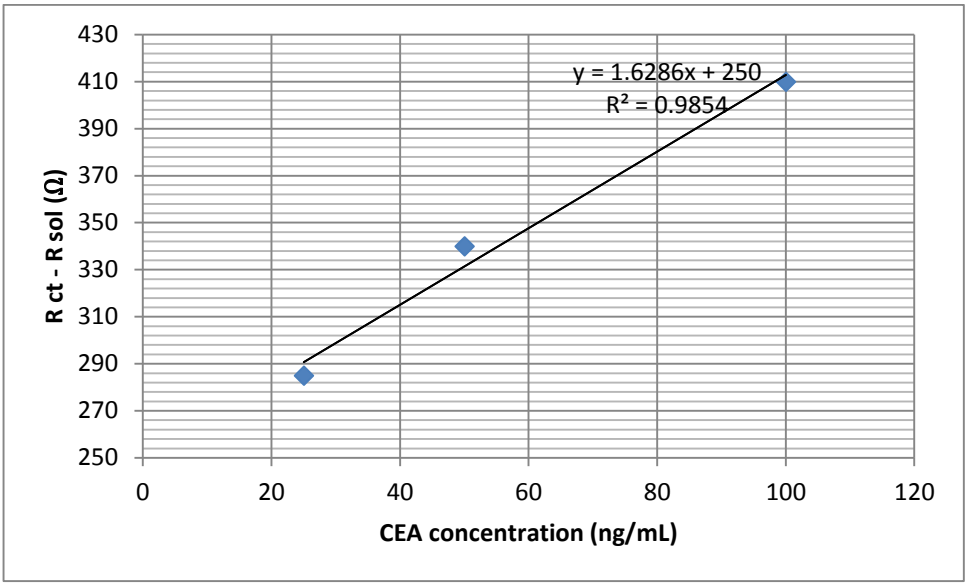

Figure 4.17 Impedance changes R_{CT}-R_{SOL} vs the CEA protein concentration interacted with monoclonal anti-CEA. All measurements were carried out in 10 mM PBS (pH 7.4) at room temperature. The impedance spectra were recorded using a sunisoidal ac potantial perturbation of 5mV (rms) in frequency range 100000-0.05 Hz, superimposed on a dc potantial of +0.14V the potantial of the redox couple ($[Fe(CN)_6]^{4-}$ / $[Fe(CN)_6]^{3-}$). R^2 = 0.9854

The corresponding calibration plot, shown in the figure 4.17, clearly implied that there was a linear relationship between the measured difference resistance of R_{CT}-R_{SOL} and 3 different CEA concentrations, with a regression equation of R_{CT}-R_{SOL}[Ω] = 1.6286 (CEA concentration [ng/ml]) + 250, R^2 = 0.9854.

5 Conclusion

In this thesis, transducer part of the biosensor were studied because Au working electrode were being used as conductor in transducer part of biosensor. Modification of Screen Printed Gold Electrode(SPGE) allows an electrochemical change on transducer of our biosensor and this changes are converted to quantifiable signals by Electrochemical Impedance Spectroscopy (EIS) and Cyclic Votagram(CV).

1,6 hexanedithiol Self Assamble Monolayer on SPGE was constructed help of extremely high affinity between gold and sulfur atoms as a linker to AuNPs adsorbtion. AuNPs facilitate electron transfer between redox proteins and electrode surfaces, provide effective mass transport in electrochemical biosensor applications as making closer redox protein (CEA Antibody) to electrode via nanosized structure. Semicircle diameter of EIS Nyquist plots impliedcharge transfer resistance, Rct and this electrochemical change are correlated with detected monoclonal CEA antibody on

AuNPs modified SPGE surface. In this study, it was implied that AuNP is a desirable intermediator for immobilization of CEA antibody as bioelement of immunosensor. Electrochemical impedimetric biosensors are more convenient to development of label free detection.

In this study, Screen Printed Gold Electrode (SPGE) was thiolated with 1,6-hexanedithiol and modified with AuNPs to increase surface area of gold working electrode.After gold nanoparticle synthesis, morphological analysis was performed with FESEM. UV-vis spectroscopy was applied and Zeta potantial of colloidal gold solution gave information about gold nanoparticles sizes. Synthesised AuNPs by Frens method produces AuNPs with reducing gold ions in $HAuCl_4$ by citrate molecules. The reduced gold atoms in solution, aggregate and form clusters, these clusters grow by increasing the number of atoms until achive the particles form. UV-vis spectrum gave about 520 nm and FESEM images implied that sythesized colloidal gold nanoparticles are about 40-45 nm.

On the gold electrode surface, the self assamble monolayer of chemisorbed 1,6 hexanedithiol has beed used as the base interface for the deposition of synthesised Au colloid by Frens method onto Au surface. Thiol group of 1,6 hexanedithiol strongly binds gold surface due to the soft character of both Au and thiol. Other thiol group of 1,6 hexanedithiol was free to bind with Au colloids introduced in solution. After surface modifications, monoclonal anti-CEAs were adsorbed on AuNPs deposited gold surface. Electrochemical characterization methods performed after each modification and anti-CEA – CEA interaction. These electrochemical characterization methods were cyclic voltametry and electrochemical impedance spectroscopy.

CEA tumor marker can penetrate to body filuid due to being on cell surface. 97% of healthy and non-smoker people have less than 2,5 ng/ml CEA in serum. 19% of excessive smokers and 7% of former smokers have more than 5 ng/ml, and this is approved as a meaningful data in the cancer phenomena. Higher level than 20 ng/ml of CEA in serum indicates metastasis stage of cancer disease and cancer prognosticate of some cancer types such colon and pancreas. CEA monoclonal antibodies were immobilized to detect carcinoembriogenic antigens. Antigens which are intended to detect are Carcinoembriogenic antigen (CEA) cancer biomarkers. Detection of CEA colon, pancreas, lung cancers biomarker from blood provides an early fast diagnose for lung, pancreas and colon cancer diseases. Because these cancer diseases are cancer

types that more insidious spread in the human body, a fast and early diagnostic is very important for cancer treatment. In this thesis, by using electrochemical characterization methods; it was aimed that CEA detection was provided on AuNPs modified SPGEs. Obtained detection of 100 ng/ml, 50 ng/ml and 25 ng/ml showed that portable SPGEs could be modified to develop a label-free impedimetric immunosensor.

In conclusion, this study implies that CEA detection on modified SPGE can be performed without labelling any molecule. Label free assesments get easier detection procedure and get shorter response time of biosensors. This can be promoted as an label free impedimetric immunosensor for follow-up care of lung cancer. Using SPGE in development an immunosensor gets easier usage of the immunosensor as portable and in pont of care. Development of a label free impedimetric immunosensor based on Ab immobilization on SPGE can lead to commercilization of these types of portable biosensor developments for other protein detections.

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PUBLISHMENTS

Poster Presentation

1. Şahin, S. Çakır, K. R. and Kır, M., "Electrochemical Impedimetric Immunosensor Based on Gold Nanoparticles Functionalized Screen-Printed Gold Electrode for Carcinoembryogenic Antigen (CEA) Tumor Marker Detection", Third International Congress on Biosensor,5-7 October 2016, Ankara, Turkey.