GEDIZ UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

ELECTROCHEMICAL DETECTION OF miR21 IN BREAST CANCER CELL LYSATES USING CONDUCTING POLYMER POLYPYRROLE, REDOX INDICATOR MELDOLA'S BLUE AND PENCIL GRAPHITE ELECTRODE

M.S.c. Thesis
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GEDİZ ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

MEME KANSER HÜCRE HATLARINDAKİ miR21' İN İLETKEN POLİMER POLİPİROL, REDOKS İNDİKATOR MELDOLA MAVİSİ VE KALEM GRAFİT ELECTROT KULLANARAK ELEKTROKİMYASAL TESPİTİ

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Merve Kaplan a **M.Sc.** student of Gediz University **Graduate School of Natural and Applied Sciences** 600713002, successfully defended the **thesis** entitled "**Electrochemical Detection of miR21 in Breast Cancer Cell Lysates Using Conducting Polymer Polypyrrole, Redox Indicator Meldola's Blue and Pencil Graphite Electrode**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission :

Date of Defense : 03 September 2015

DEDICATION

To my family

FOREWORD

First my earnest gratitude goes to my advisor in person of Prof. Dr. Mehmet Emin Şengün Özsöz for his all efforts for the success of this thesis. I really am greatful for working with him because his recommendations and supports motivated me to go further in my academic carrier.

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September,2015 Merve KAPLAN

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ABBREVIATIONS

Ɵ : Phase angle

ABS : Acetate Buffer Solution

Ag/AgCl : Silver/silver Chloride

BC : Breast cancer

Cdl : Double layer capacitance

CE : Counter electrode

CLL : Chronic lymphocytic leukemia

CM : Circulating miRs

CV : Cyclic voltannetry

DGCR8 : DiGeorge syndrome chromosomal or critical region 8

DNA : Deoxyribonucleic acid

DPV : Differential pulse voltammetry

ds-DNA : Double stranded DNA

EIS : Electrochemical impedance spectroscopy

Exp -5 : Exportin 5

HCC : Hepatocellular carcinoma

I : Current

I (*t*) : Circuit the current

$K_3[Fe(CN)_6]$: Potassium Ferri cyanide

$K_4[Fe(CN)_6]$: Potassium ferro cyanide

KCl : Potassium chloride

LOD : Detection limit

MCF-7 : Michigan Cancer Foundation-7

MDB : Meldola' s Blue

miR : microRNA

NADH : Nicotinamide adenine dinucleotide

NC : Nucleic Acid

PBS : Phosphate Buffer Solution

PCR : Polymerase Chain Reaction

PGE : Pencil graphite electrode

PPy : Polypyrrole

pre-miR : precursor RNA

pri-miR : Primary miR

Py : Pyrrole

R : Resistance

R_{ct} : Resistance Charge transfer

RE : Reference electrode

RISC : RNA-induced silencing complex

RNA : Ribonucleic acid

R_s : Solution resistance

SELEX : Systematic Evolution of Ligands by Exponential

ss-DNA : single stranded DNA

TBS : Tris Hydrochloride Buffer Solution

$TGBRF_2$: Transforming Growth Factor, Beta Receptor II

UTR : Untranslated region

V : Potential

WE : Working electrode

Z : Impedance

Z' : Real resistance

Z" : Imaginary resistance

ZnO : Zinc oxide

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SUMMARY

In the present work, an electrochemical nucleic acid biosensor has been designed for the purpose of detection of miR21 in real samples, i.e cell lysates. The fabrication of biosensor has three steps choosing the right target, immobilization method and detection methods. The probe of the upregulated miR21 in breast cancer was determined antimiR21. Entrapment of antimiR21 onto pencil graphite electrode (PGE) was chosen as the immobilization methods for the study. Solid phase hybridization with either synthetic miR21 or miR21 included in total RNA isolated from MCF-7 cell line. MCF-7 contains large amount of upregulated miR21 also in order to assert the antimiR21 is specific to the miR21 HUH-7 cell lines were used. HUH-7 cell line comprises of rich amount of miR122. The characterization of Polypyrrole (PPy) modification onto the PGE surface has been proved via, Electrochemical impedance spectroscopy (EIS) and differential pulse voltammetry (DPV). Hybrid signal measured via DPV and the charge transfer resistance, R_{ct} values recorded via electrochemical circle fit option of EIS have been used. Conducting polymer, PPy enabled not only very low detection limit but also more effective immobilization of antimiR21. PPy solution was prepared using acetate buffer solution (pH:4.80). PPy was achieved by using repeated cyclic voltammetry from pyrrole solution.

At the first step the cycle number was chosen. At the second step parameters such as the pH, immobilization method, probe concentration, hybridization time, target concentration were optimized. It was observed that cycle repetition number was 5, pH of the pyrrole solution was 4.80 , the probe concentration was 5 µg/ml, the target concentration was 7µg/ml, hybridization time was 30 minutes, immobilization method was found the entrapment of the antimiR21.

Electrochemical studies of miR21 was achieved using redox indicator Meldola's Blue via DPV and redox probe potassium Ferri cyanide ($K_3[Fe(CN)_6]$)via EIS. The PGE coated with PPy and the bare electrode R_{ct} values were compared and it was found that the PGEs coated with PPy provided 4 times lower R_{ct} values than bare PGEs. Electrochemical detection of miR21 was carried out using a non complementary target, full match target and mismatch target. It was clearly shown that the antimiR21 is specific to the miR21 and even with the mismatch sequences of

the miR21 it was possible to gain a response. Also each electrode showed great stability.

The proposed biosensor with the detectionof 1.4 pmol is applicable for analysis of certain microRNAs as well as miR21 from total RNAs isolated from cell lysates.

ÖZET

Bu çalışmada hücre hatların gibi gerçek örneklerde miR21' in tespiti için bir elektrokimyasal nükleik acid biyosensorü tasarlanmıştır.Biyosensörün üretiminin doğru hedefi, immobilizasyon ve tespit yöntemini seçmek üzere üç basamağı bulunmaktadır. Meme kanseride up regüle olan miR21 için hedef olarak antimiR21 seçilmiştir. Katı faz hibritleşme sentetik miR21 ya da MCF-7 hücre hatlarından elde edilen total RNA' daki miR21 seçilmiştir. MCF-7 fazlaca miktarda up regüle olmuş miR21 içermektedir. Ayrıca antimiR21' in sadece miR21'e özgü olduğunu ispatlamak için HUH-7 hücre hattı kullnılmıştır. HUH-7 zengin miktarda miR122 içermektedir.Polipirolün (PPy) PGE yüzeyine karakterizasyonu elektrokimyasal impedans spektroskopisi (EIS) ve differensiyal puls voltametrisi (DPV) ile kanıtlanmıştır. Hibrit sinyali DPV ve yük transfer direnci (R_{ct}) ile ölçülmüştür. R_{ct} değerleri elektrokimyasal daire fit ile belirlenmiştir. İletken polimer,PPy sadece çok düşük bir tayin sınırı sağlamakla kalmamış ayrıca antimiR21' in daha etkili bir biçimde imobilizasyonu sağlamıştır. PPy çözeltisi asetat tampon çözeltisi (pH:4.80) kullanarak hazırlanmıştır. PPy, pyrrole çözeltisinden tekrarlı döngüsel voltametri kullanarak elde edilmiştir

İlk basamak olarak döngü sayısı belirlenmiştir. İkinci basamakta pH, imobilizasyon yöntemi, prob derişimi, hibritleşme zamanı, hedef derişimi gibi parametreler optimize edilmiştir. Döngü sayısı 5, pirol çözeltisinin pH 4.80, prob derişimi 5 μg/ml, hedef derişimi 7 μg/ml, hibritleşme süresi 30 dakika, imobilizasyon yöntemi antimiR21' in hapsolması olarak bulunmuştur.

miR21' in elektrokimyasal çalışmaları DPV kullanarak redoks indikatörü Meldola mavisi, EIS kullanarak redoks probu potasyum ferri siyanür ($K_3[Fe(CN)_6]$)ile gerçekleştirilmiştir. PPy kaplı PGE ve çıplak PGE' nin R_{ct} değerleri karşılaştırılmıştır ve PPy kaplı PGE 4 kat daha az R_{ct} değerine sahip olduğu gözlemlenmiştir. miR21' in elektrokimyasal tespiti koplimenter olmayan hedef, tam olarak eşleşen hedef ve uyumsuz hedef ile gerçekleştirilmiştir. Açıkça gözükmektedir ki antimiR21, miR21' e özgüdür ve uyumsuz dizilerde bile miR21'e ait bir cevap elde edilmektedir. Ayrıca herbir elektrot oldukça iyi tutarlılık

Önerilen biyosensör 1.4 pmol tayin sınırına sahiptir ve bu değer sentetik ve total RNA' lardan elde edilen mikroRNA'lar için olduça uygundur.

1.**INTRODUCTION**

Breast cancer (BC) is a leading cause of cancer death among women in industrialized countries, despite advances in early detection and treatment. BC is the most common invasive cancer in females worldwide. It accounts for 16% of all female cancers and 22.9% of invasive cancers in women. 18.2% of all cancer deaths worldwide, including both males and females, are from breast cancer.

In the last decade, technological advances, particularly in genomics and proteomics, have offered the possibility of improving treatment seam through the identification of new targets for cancer therapy. These molecules are involved in several processes relevant to BC biology, such as signal transduction, cell cycle, apoptosis, pro-angiogenic and metastatic pathways. These new potential diagnostic and therapeutic targets, it is diagnosed by microRNAs (miRs) that are considered abundant class of short noncoding RNA from about 17 to 25 nucleotides, which contain a seed sequence for binding to the 3 'untranslated region of the mRNA specific target for regulating biological events, including individual development, apoptosis, proliferation and differentiation .

They are also associated with important human diseases involving the immune system, as well as neurologic diseases and infection. Recently, miR expression profiling, using oligonucleotide miR microarray analysis, has revealed cancer-specific miR deregulation in human tumors. MiRs can act as tumor promoters or suppressors. Therefore, deregulated miR expression and its molecular basis have been important subjects for research in recent years .

These small, non-coding RNA molecules act as transcriptional repressors by binding to the three prime untranslated region (3′UTR) of target mRNAs .

The differential expression of miRs might have potential clinical value as biomarkers or treatment targets in the early diagnosis, treatment, and prognosis of many carcinomas.

In this report, Polypyrrole (PPy) modified pencil graphite electrodes (PGEs) were designed for the purpose of specific recognition of miR-21, which was related to breast cancer disease .

Polypyrrole, is still the one of the most studied polymers. Especially, in electrochemical approach , is commonly used as suitable substrates for immobilization of biomolecules and can be used to enhance flexibility, ease of processing, low toxicity, stability, speed, and sensitivity of biosensors. PPy is the

most conducting polymers suitable for electrochemical DNA sensors because it acts as linking agent for the immobilization of the DNA probe and insures efficient electron transfer between the transducer and the electrode surface .

Our purpose in this study is to advance fast, economic, label-free and sensitive biosensor, in order to detect microRNAs at low concentrations based on Meldola's blue reduction signal and resistance charge transfer.

The probe was immobilized by electropolymerization of pyrrole on the PGE surface. The target was immobilized by physical Adsorption on the same electrodes. The Hybridization time is 30 minutes. All the stock solutions were prepared daily in Phosphate Buffer solution. Hybridization of the immobilized probe with the target at the electrode surface was monitored by Electrochemical Impedance Spectroscopy (EIS) and Differential Pulse Voltammetry (DPV) by using Meldola's blue (MDB) as an electrochemical indicator with the application of an electrode potential of vs. Ag/AgCl.

The most suitable probe concentration was found to be 5 μg/ml for each electrode. It is clearly observed from the results based on the Meldola's blue that the microRNAs form easily the hybrid.

This method shows great feasibility for the Detection of microRNA and may have potential applications in cancer diagnosis and prognosis based on the results already found. It is acceptable to use 1.4 pmol as concentration limit in certain concentrations of microRNAs. This Electrochemical biosensor has essential characteristics such as low-cost, rapid, sensitive and no need for purification.

2. BIOSENSORS

2.1. History and Discovery

DNA biosensors or genosensors have caught great attention as point-of-care detection tools. Biosensors provide rapid, sensitive and low cost way diagnosis of diseases owing to their specificity to DNA sequences [1]. The definition of an electrochemical biosensor according to the International Union of Pure and Applied hemistry 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]. The first biosensors came into

being in 1956 by L.C.Clark who is assumed to be the father of the biosensing field. The biosensors that he developed were aiming to detect glucose levels in an enzyme-based approach [3].

Biosensors consist of two elements; physicochemical transducer and a recognition surface. From the interaction between biorecognition surface/electrode/transducer 1 and the analyte, a biochemical response occurs. This response is measured by the transducer and translated into a phsysical signal such as pH, temperature (Figure 1)

Enzymes, antibodies, nucleic acids (NA) are used as biorecognition surfaces. The classification of biosensors can also be done according to the measurement techniques such as:

1. Electrochemical Biosensors

   - Potentiometrical Biosensors

   - Amperometric Biosensors

2. Piezoelectric Biosensors

3. Optical Biosensors

4. Colorimetic Biosensors

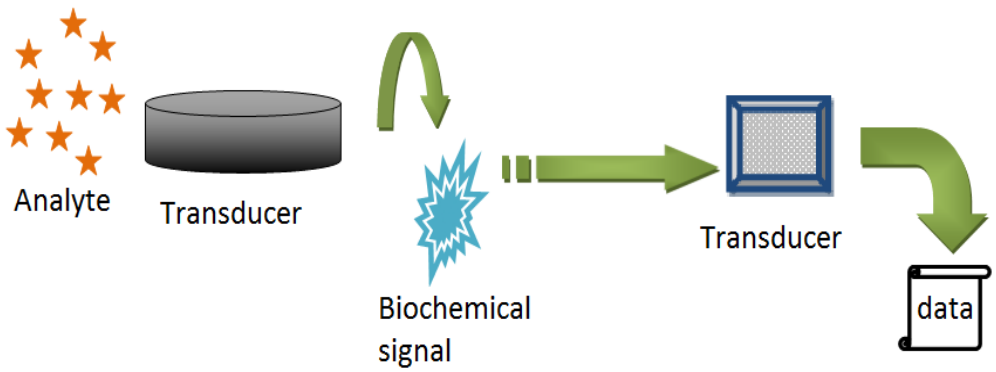

Figure 1: Schematic presentation of biosensor

2.2 Bioreceptors

2.2.1 Enzymes

Enzymes are one of the most well known bioreceptors. It is convenient to use enzymes due to their affinity to specific substrates. Beside biosensors, enzymes are important in speed, pH or heat of the reactions.

2.2.2 Antibodies

Antibodies are y-shaped-glycoproteins that have two antigen recognition region and are specific to the antigens. The Binding between antibody and antigen is reversible, cite-specific, pH, temperature and ion-dependent. The antigen is the response against to the foreign molecules in the organism.

2.2.3 Aptamers

An aptamer is a short piece of DNA that binds to a target firmly. They can be synthesized using Chemical or molecular biological methods. Systematic Evolution of Ligands by Exponential (SELEX) is the method in order to find an aptamer. Briefly in SELEX, Random sequences of DNA are introduced to the target, some of DNAs interact with the target some don't. After multiple times of Ploymerase Chain Reaction (PCR), the possible best strand of the DNA is found for the target. After final selection of the strands interact with the target, the best DNA strand is selected.

2.2.4 Proteins

Proteins are bioChemical composite that is formed by folding . Aminoacids are the building blocks of the proteins. Amino acid is connected chain-formed. Every protein has specific sequences that make them unique. This uniqueness makes proteins of the most popular bioreceptors in biosensors.

2.2.5 Nucleic acids

Nucleotides consist of 3 main parts: 5 carbon sugar, phosphate groups and bases as seen in Figure 2.

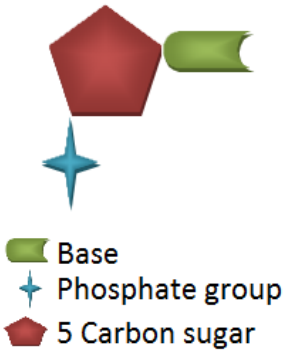

Figure 2: Schematic structure of a nucleotide

2.2.5.1 Deoxyribonucleic Acid (DNA)

DNA consists of deoxyribose, bases, a phosphate group. The bases are Guanine, Adenine, Thymine, Cytosine. The bases always bind the first carbon of the sugar. DNA shape reminds a staircase in tangled. Each half wraps up the other one. DNA is double stranded. The reason behind calling the sugar deoxy is that it doesn't have any oxygen in second carbon in its pentagon structure. At a third carbon there is a phosphate group and this group give the DNA negative charge. DNA replication is based on sequence specific Binding between bases. For example Adenine binds to Thymine and Cytosine binds to Guanine. Between Adenine and Thymine there is two weak hydrogen bonds on Guanine - Cytosine there are three weak hydrogen bonds. Any reaction in DNA takes place from 5' to 3'.

2.2.5.2 Ribonucleic acid (RNA)

Ribonucleic acid (RNA) consists of nucileotides. Like every nucleic acid (NA), it has a base, ribosee sufar, phopsphate group.RNA is usually single stranded, but in rare cases it is double stranded. Like DNA it has sugar, bases and a phosphate group. The sugar is called ribonucleic acid. But there is a slight change in the bases instead of Thymine there is Uracile. RNA has a hydroxyl group on the second carbon of the sugar. The placements of a phosphate group and bases is just like DNA's.

RNA plays important roles in many biological proccesses such as protein Synthesis. RNA and DNA are similar yet they have slight differences. RNA is single stranded but DNA usually is double stranded. RNA consists of Ribose but DNA consists of deoxyribosee sugar. Instead of Thymine RNA has Uracil as base.

The first DNA biosensor was presented by Palecek et. al. in 1960s where the Electrochemical nature of nucleic acid, was shown. Since then, various studies have been performed .The basis of electrochemical genosensors is the detection of hybridization between target and probe nucleic acid sequences by various electrochemical techniques suc as differential pulse voltammetry [6], square wave voltammetry [4], cyclic voltammetry [5], electrochemical impedance sprectroscopy [6], chronoamperometry [7], chronopotentiometry [8]. The detection of hybridization has been achived by different techniques that can be subgrouped into two as label-based and label-free techniques which will be detailed in section 3.3.1. These techniques are applied in order to get a signal from ss-DNAs or ds-DNAs using oxidation of bases. There are four bases in DNA.: Guanine, Adenine, Thymine, Cytosine. In RNA, There are Uracile bases instead of Thymine bases. Each base has different oxidation voltage. Adenine and Guanine bases are oxidized at solid electrode but Thymine, Cytosine and Uracil is not capable of being oxidized at solid electrodes.

Yu-Zhi Fang et. Al. , 2003 describes the Electrochemical techniques as inexpensive, delicate, formed from different disciplines, not occupying time techniques. There are quite techniques for the Detection of DNA, but Electrochemical Detection technique has important benefits, such as simple, rapid, sensitive, label-free, inexpensive and suitable for other biomedical detection. Very low concentration, low Detection limit (LOD), working with various electrodes are allowed. Electrochemical methods are stable methods for detection of biomolecules and allow the miniaturization of devices for example, glucose in the blood can be detected very small devices and one person can detect the amount of glucose in their blood easily without Using a very large amount of blood.

2.3 Electrochemical Biosensors

In electrochemical DNA biosensor, it is important to observe the differences of peak currents between single stranded DNA and double stranded DNA. Many detection techniques use a single stranded DNA and its target in order to determine efficiency of the biosensor [9]. There are quite amount of electrochemical methods for the biosensors such as differential pulse voltammetry, cyclic voltammertry, chronoamperometry, square wave voltammetry, chrono-potentiometery etc.

Electrochemical DNA sensors have more benefits compared to the other sensors due to their simplicity user-friendliness, low cost, suitablity for mass production, portability, sensitivity and selectivity . Also DNA biosensors have wide variety of use like diagnostics, environmental monitoring, criminal investigations, food industry and detection of warfare agents.

2.3.1 Electrochemical cells

Measurement cell is the place where all the electrochemical reactions and measurements happens. Electrochemical cell can be designed in different volumes and shapes depending on the nature of the study. Mostly cells are made of glass, but if a spectrochemical experiment is needed to be studied the cell material should be able to transfer the light easily considering the fact that a light reflection could be observed due to electrochemical signal change.

2.4 Electrode types

In electrochemical setup mostly three electrode system is used these electrodes are counter electrode, working electrode reference electrode.

- Working electrode
- Counter (auxiliary) electrode
- Reference electrode

2.4.1 Reference electrodes

In many electroanalytical studies , it is desired that one of the electrodes in the system is not affected from the solution. It is called as reference electrode (RE). The first electrode that is used with reference electrode is indicator electrode, the second electrode move according to the ions concentration in the solution.

There are some factors that needs to be considered when a RE is used:

- Concentrations of ions must remain same.
- Must not be affected by the experiment solution, ions, potential, change of the current
- Must be polarized
- The reaction in the reference electrode must be reversible

There are many different types of REs. For instance a standard hydrogen electrode meets factors above, but in practice it is hard to use hydrogen electrode as a reference electrode. Silver/silver Chloride (Ag/AgCl) reference electrodes are used frequently. Ag/AgCl reference electrodes comprise of an Ag wire (%99. 999 purity) coated with a saturated Potassium chloride (KCl) solution. (Figure 3)

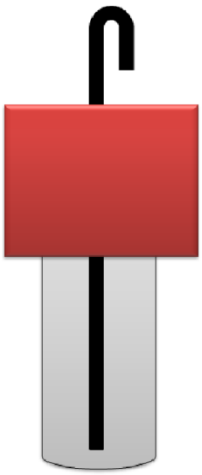

Figure 3: Schematic of reference electrode

2.4.2. Working electrodes

In electrochemical studies, there are various types of working electrodes. Some of these working electrodes are shown in the Table 1.

Table 1: Classification of working electrodes

Carbon based electrodes	Solid Metallic electrodes	Mercury based electrodes	The other electrodes
Pencil Graphite	Gold	Hanging mercury drop	Composites
Glassy carbon	Platinium	Amalgam electrodes	ITO (Indium tin oxide)
Carbon paste	Bismuth	Dropping mercury	Rotary spinning electrode
Carbon Fiber			

The working electrode (WE) is the electrode that the analyte is studied. WE needs to to have some properties such as stability and easy preparation. The WE can

be solid or liquid. For instance Pencil Graphite Electrode (PGE)is a solid working electrode with easy preparation and low cost and hanging mercury drop electrode is liquid working electrode with unaffectiveness of depositing metals.Various studies have been done with different working electrodes of Graphite [10, 11], carbon paste [12], glassy carbon[13], gold [14], microarrays [15].

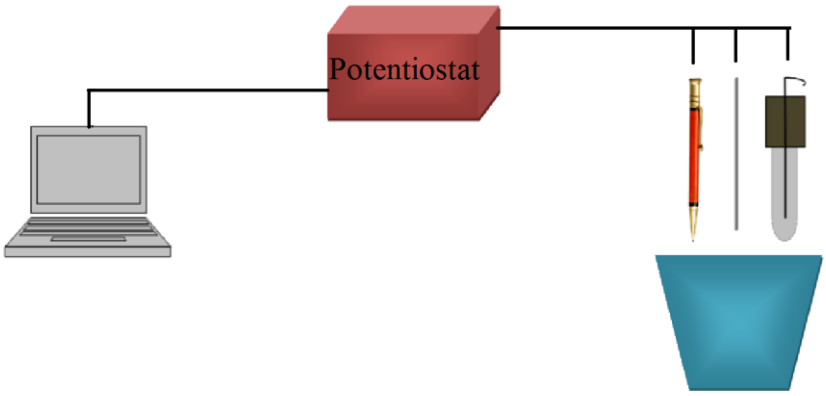

Figure 4 : Schematics of conventional three-electrode-system connected to potentiostat and personal computer

2.4.3.Counter (auxiliary) electrodes

In electrochemical measuements, potential is controlled and the aim is observing the current , generally WE signals form in a wrong way. In order to overcome this problem, another third electrode (counter electrode,CE) is needed (Figure 4). Current flows through from WE to CE. WE potential is measured under nearly zero potential. CE does not affect the reaction in the electrochemical cell also need to be larger than WEs and kept near the WE. As a CE platinum, tantalum, tungsten, carbon wires have been used.

3. DESIGNING OF AN ELECTROCHEMICAL DNA BIOSENSOR

The main steps to design a biosensors are: Choosing the gene sequence, Immobilization, Detecting Hybridization.

3.1 Immobilization

Immobilization of DNA onto electrode can be achieved in many ways(Figure 5) such as:

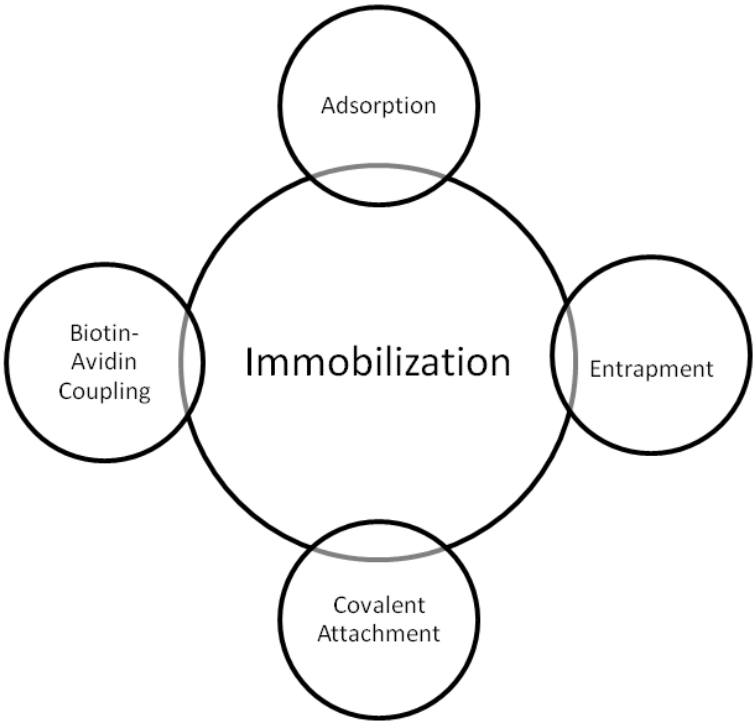

Figure 5: Immobilization methods

3.1.1 Adsorption

A kind of physical binding where fluid phase of certain components in dissolved form holds on to a solid adsorbents based on surface adhesion.

Mainly three kind ways of adsoption are observed and these are seen together or sequentially in many Adsorption cases :

3.1.1.1 Physical adsorption

It is formed by van der Waals forces between solid surface and adsorbent. Physical Adsorption is reversible and there might be heat as result of condensing energy.

3.1.1.2 Chemical adsorption

Chemical Adsorption is comprised of attraction between functional groups of solid surface and adsorbent. It is irreversible and Adsorption released heat is bigger than reaction heat. : Electrode is coated with monomoleculer layer generally

3.1.1.3 Ionic adsorption

With the electrostatic forces, ions are held on to the charged areas on the surface. In ionic Adsorption it is important to noted that between adsorbent and adsorber ions , smaller charged ions are absorbed

3.1.2 Covalent attachment

It depends on engaging, functional groups to the bare electrode. There is a covalent bond with the functional group and the substrate . At first Murray et. al. in 1978 used this techinque and they interacted hydroxy groups on the electrode surface and organo cylane. Carbon based electrodes are avaible to create functional groups on their surfaces. Especially with Glassy carbon electrode there are lots of studies about covalent attachment.

3.1.3 Entrapment

This will be explained in detail in section 5.6.2

3.1.4 Biotin-Avidin coupling

Biotin has a strong affinity (Ka: 10^{15} M^{-1}) to avidin. For sensing purposes, avidin is coated onto the electrode surface and biotin linked DNA/biotinylated DNA is introduced to the electrode. Biotin-avidin coupling creaes strong bond.

3.2 Electrochemical Measurement Techniques

3.2.1. Cyclic voltammetry

Cyclic voltammetry begins in 1938. It is usually used in order to understand the nature of the electrode surfaces. In this techniques scanning takes place in two different potential ranges with the linearly changing potential. The electrode is scanned in forward and backwards way. After scanning two peaks are observed: the

oxidation peak and the reduction peak. By using CV the features below could be found:

- Diffusion controlled or not
- Adsorption
- If cycle number is more than one, nature of the mechanism

It is important to choose initial potential where any redox reaction does not occur. The electrode is scanned first forward and second backwards potential limits with a certain scan rate.The scanning end at the stop potential. All these potentials and scan rate differs according to the material usage purpose.

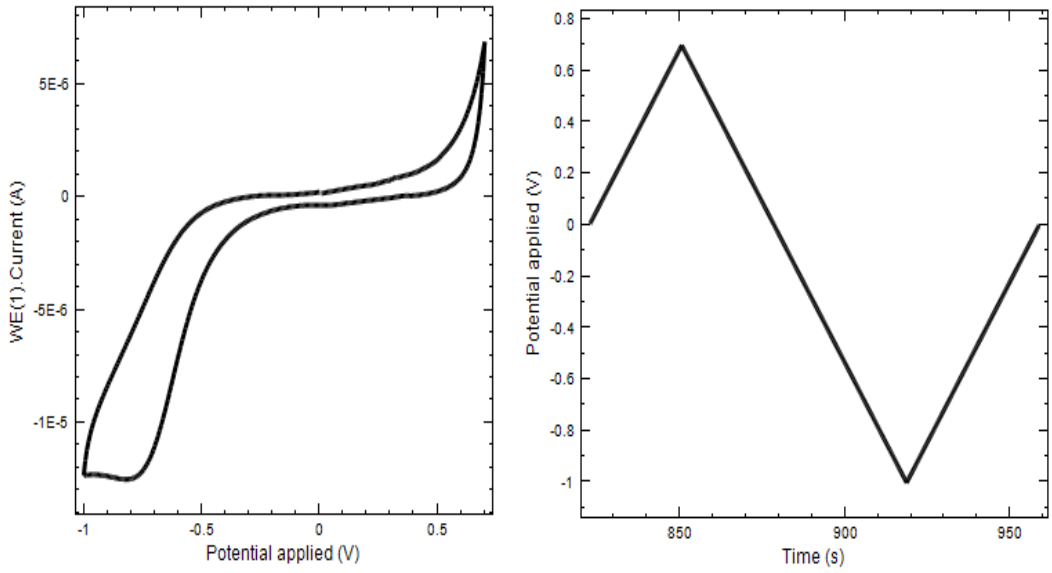

Figure 6: Typical potential scan in cyclic voltammetry

One of the parameters , scan rate affects the results. GenerallytThe scan rate is between 1 mV/s and 1 V/s. But when scan rate get close to the 1 V/s there might be some problems such as double layer capacitance.

In reversible reactions anodic and cathodic peak potential difference needs to be 59 mV and it shows that it is adsorbtion controlled reaction. In inreversible reactions this difference more or less than 59 mV and peak shapes are not define well. Anodic and cathodic peak heigth is different. If a reaction completely inreversible then reduction peak cannot be observed.

CV is also used in adsorption and desorption. If anodoic and cationic peaks current is increased gradually, it is a sign of adsorption. If product gathers around the electrode surface then forward peak is observed at posivitive potentials.

3.2.2. Electrochemical impedance spectroscopy

Ohm's law explains the relationship between current and potential. Ohm's law is presented via equation as

$$V = I.R.$$

(1)

Each interface stores charge and conducts the current. At the same time there is a potential difference in each interface. But only the results of WE (E_1) and solution interface changes against the potential applied. In order to get better results it is important to choose the right CE (E_2). The CE needs to be nonpolarized by the reason of decreasing potential between CE and solution interface measurement results.

In a system like above all the impact that causes resistance against to the current is called impedance (Z). The word impedance comes from impeding. Basicly impedance is based on measuring the resistance originated from changes between capacitance and inductive when high frequencies are applied. Impedance spectroscopy is defined as the impedance variation due to certain frequency range. Frequency is applied to the system as sinusoidal waves and related to the alternative current. This makes the amplitude of sinusoidal wave valuable and E_1 and solution interfaces becomes dependent on the time.

In a circuit the current against alternative potential is in sinusoidal form and represented as I (*t*). It is well explained in the equation below [16]:

$$I(t) = Ipeaksin(\omega t + \theta)$$

(2)

Θ is the phase angle and related to the storing properties of solution interface. It needs to be noted that interface stores charge due to its electrical double layered and capacitive nature. When a charge is transferred from an interface, interface stores

charge, then transfer the charge and this causes a phase difference between potential and alternative current. The total equivalent resistance than corresponds to the interface is also called as impedance. It is presented as the following equation[17]:

$$V = I.Z \tag{3}$$

$$Z = Rs + \frac{Rp}{1 + (\omega CdlRp)^2} - j\frac{\omega CdlR^2p}{1 + (\omega CdlRp)^2} \tag{4}$$

According to the equation (4), The resistances are two parts besides the solution resistance (R_s). First part is the real resistance (Z') and the other is imaginary resistance (Z''). The reel resistance stands for the resistance that current pass through. Z'' depends on alternative current frequency.

The graphic between Z' and Z'' is called Nyquist graphic (Figure 7) and the angle of these two parameters is the phase angle.

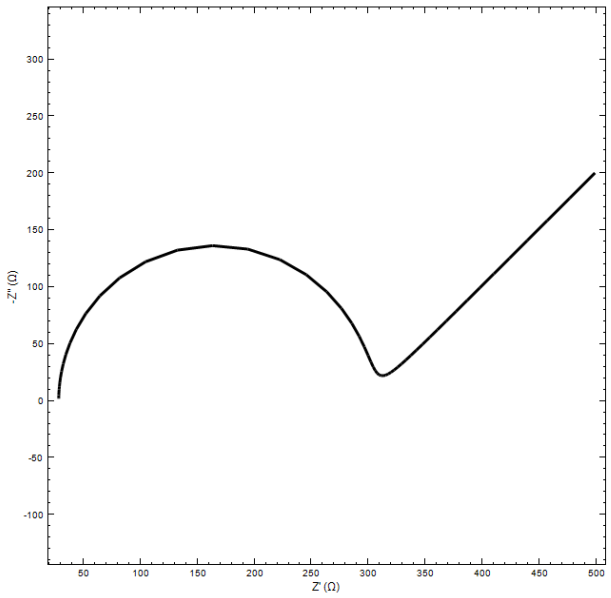

Figure 7 : The Nyquist curve

During EIS process the form of the semicircle is observed at higher frequencies and linear line is observed at lower frequencies. Linear part presents the

diffusion known as Warburg Impedance. These semicircle or linear lines at higher frequencies presents resistance charge transfer (Rct) values of material or electrode. Rct depends on the electrostatic attraction between ions in the electrochemical cell and analyte [18]. If the linear part doesn't exist in the graphic , Rct value , also the diameter of semi circle, is too high and there is so much probe on the electrode [19].If the sample behaves like diffusion controlled, the linear line is monitored instead of semi circle at higher frequencies. Also the bode graphic is drawn between impedance and frequency or phase angle and frequency (Figure 8). Resistance and capacitance transfering , frequencies, double layer capacitance values, corosion properties could be found using bode graphics.

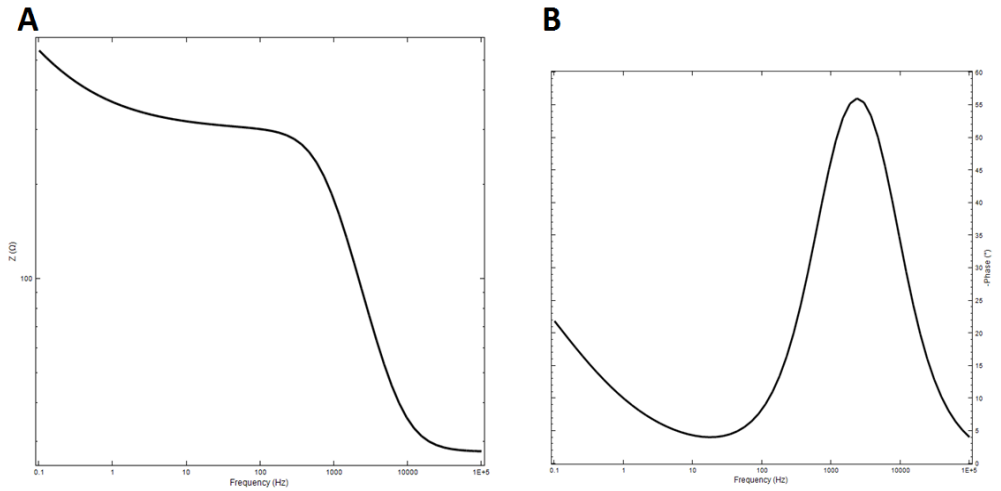

Figure 8: The bode graphics. Frequency and impedance (A) , frequency and phase (B)

Electrochemical impedance is studied in the Potassium Ferri cyanide ($K_3[Fe(CN)_6]$) and Potassium ferro cyanide ($K_4[Fe(CN)_6]$) solutions. Mostly hybridization is studied with ferro/ferri cyanide or ferricyanide in biosensors using EIS [20]. As a redox probe Ferri/ferro cyanide performs very effectively for the biosensors and presents very low potential. If the hybridization is successful, the Rct values would be affected by it [21].

EIS is used in biosensors in order to monitor the hybridization by using the changes of Rct values. The hybridized probe and target or alone target and probe cause the decreasing of the double layer capacitance therefore dielectric constant reduces. Additionally, the flow of the current could be prevented As a result Rct

values increase [22]. Because of these reasons solution resistance, diffusive capacitance and double layer capacitance are important in EIS while studying biomolecules [23]. Before studying EIS, layer preparation of the electrode is significant. The layer should be formed properly and compitable with the biomolcules. Otherwise the results are not sensitive and effective [23] .

EIS has a wide range of usage areas such as corosion, biosensors, metal coating, thin films, bataries, semiconductive polymers [24]. The EIS is a useful method to study high resistance samples. Most importantly, it does not damage the analte on the electrode surface.

3.2.3 Differensial pulse voltammetry (DPV)

In DPV signals are occured during lineer scannig**.** Constant high pulses are applied to the working electrode for a while. Current is measured twice. First , at the same time of the starting of the pulse without increasing(16.7 ms), second right before the end of the pulse(16.7 ms) [25]. Difference between two pulses is named as ΔI pulse. After measurement V vs. I graphic is observed.

DPV have quite low limit of Detection (LOD) and even the smallest differenced between two currents peak can be measured. Measurement is done the moment when faradaic current is high and capasitive current is the lowest.

3.3 Hybridization Detection Techniques

Hybridization has important place in biosensors. According to the hybridization detection results, it can be decided whether biosensor works or not. Hybridization is performed either solid phase or liquid phase. Hybridization is more succesful Using liquid phase rather than solid phase. Because in liquid phase target and probe are in the same enviroment yet in solid phase requires sensitive conditions . hybridization are observed two main methods. These methods are given in detail below

3.3.1. Label-free techniques

NAs are active Electrochemically due to oxidizing and reducing nature of the bases. That is why using bases properties hybridization is detected easily.

3.3.1.1 Label-free method based on Guanine oxidization

Guanine is oxidized approximately at +1.0 Volt(vs. Ag/AgCl). Also Adenine is oxidized at+1.2 Volt. But Adenine presents low repeability so mostly Guanine is used in order to detect Hybridization. Due to mathced bases in Hybridization, oxidation of double stranded (ds-DNA) Guanine signal is lower than single stranded (ss-DNA).

3.3.1.2 Label-free method based on Inosine

Inosine is a nucleosid that is binded to Cytosine with two bonds. Also Inosine does not present oxidatio signal. Due to thsi property of Inosine, hybridization is detected [26]. Also this system is known as Yes or No system.

3.3.2 Label-based techniques

NAs have very unique properties yet in some cases it not enough. Redox indicators are avaible for cases like this. Redox indicatiors are binded either ss-DNA or ds-DNA. Redox indicators performs in DNA damage studies, Hybridization studies.

3.3.2.1 Redox indicators binded single stranded DNA

Methilene Blue is the most known indicator. Methilen blue interacts with Guanine compunds. Due to matched Guanine in hybrid, Methilene blue binded to ds-DNA presents lower signal than ss-DNA.

3.3.2.2 Redox indicators binded to double stranded DNA

Meldola's Blue is the typical example for this indicators. Intercalators such as Meldola's blue, methylene blue, is used to observe interaction probe and target DNAs. 8- dimethylamino-2,3-benzophenoxzine, briefly MDB is a redox dye containing naphthalene group [27]. MDB is benzene structured redox dye. It belongs to the phenoxazin dye family, which means it can be both oxidized or reduced. It can be studied wide range of pH (pH: 3-10) and electrodes. It is biocompatible and intercalating between ds-DNA [28]. It can be studied with amperometric or potantiometric methods also lately Raman Spectrocopy is one of the attractive method [29]. Signal of MDB is gained at the potential of aroun -0.17 V at pH:7 [28].

In reduction of MDB there are two single electroned hydrogens and they seem to be positive charged but they are actually negative charged. First hydrgene breaks the bond between the ring and nitrogen where methyl groups are binded. The electrode in the middle changes place and goes to ring but it aims to form the most stabil structure. Nitrogene in the ring breaks the its double binds and the 2 electrones in the ring are places as one is outside the other one is inside. The second hydrogen attaches to the nitrogen. In brief electrones are in the bonds forms new bonds (Figure 9).

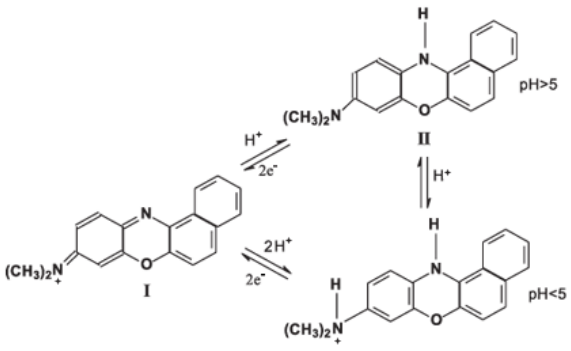

Figure 9 : Mechanism of MDB reduction [30]

Main usages of MDB are divided in two categories: to create a stable platform for the probe and to observe the DNA Hybridization. MDB is studied with a variety of samples. The most common one is the NADH sensors. To detect nicotinamide adenine dinucleotide (NADH) MDB has been studied with Zinc oxide (ZnO) crystalline**,** MWCT to provide perseverance of electrode against to the ethanol or lactate with quite sensivity [31, 32]. **A**lso it is feasible to study hydrazine, β-D-glucose, protein activity pestisides, hydrogenperoxide**,** gene mutations [33]**.**

The commercial first biosensor is enzyme biosensors in another name Glukose Oxidase Electrode [3]. It leads to a huge breaking through in the biosensor field. From medicine to defense, especially in industry, biosensors make people understand their importance.

4. microRNAs

4.1 Introduction

RNA-RNA complexes has important part in gene expression [25] because that reason miRs have been studied extensively. In genome 1/3 of RNAs were considered as junk after studies about RNAs , it has become considerebly important and begun to be understood their part in biological proccesses. In 1993 Victor Ambros and his collegues noticed some defects in Caenorhabditis elegans. After intense studying for a long time, it was found that instead of producing proteins,lin-4 gene produced some small RNAs. These RNAs were 22 nucleotide long and turned out to be a huge event. Because it is well known that approximatly 60-80% of RNAs are translated into the proteins.The small RNAs kept their mystery until the early 2000s. After explaining the mechanism of RNA interferance by Fire and Mello, the small RNAs are called microRNAs (miR). With this discovery a new, promising door has been opened to the science. Still the microRNA mechanism has lots of things to be discovered.

Briefly miRs are are small, non-coding, 22 nt long epigenetic agents. miRs have important parts in apoptosis, embrionic cell growth, cell proliferation, gene regulation, cell differention, inflammation etc. At first miRs were considered to have affinity to only one gene but today it is known that miRs can regulates more than one mRNA expression and mRNAs can regulates more than one miR[34] which means miRs could have hundreds of targets and the exact opposite is possible. miR deforms structure of mRNA as a result of regulation of proteins and genes are altered and all those changes result in diseases. Also miRs beheave like oncogemic or oncogenic suppressor [35].

miRs can be detected using microassays, nothern blotting, real time PCR but these analysis techniques have some limitations such as time consuming, cost money, requires complex instruments, radioavtive probe usage, necessity for pre-concantrate. For example Nothern blotting needs radioactive label and lots of samples. Also its high detection limit (LOD, 10^9 M) makes this techniques inconvenient [36]. Yet the electrochemical detection stands out among these techniques due to possesing considirable benefits low cost, short analyzing time, requirable sensivity.

4.2 Biogenesis of microRNAs

Biogenesis of miR has three basic steps:

- Nuclear Processing
- transport
- cytoplasmic processing (Moore et al, 2013)

The biogenesis of miR starts with transcription of DNA. DNA is trancripped to primary miR (pri-miR) which pri-miR has 5' cap and poli(A) by RNA polymerase II enzyme from introns of genes. pri-miR has 500-3000 bases long hair-pin structure.

Pri-miR is transformed into 70 nt long precursor RNA (pre-miR) by Drosha and DiGeorge syndrome chromosomal or critical region 8 (DGCR8) complex. Drosha is from RNA polymerase III family and has three parts ; RNA-Binding region and two RNA polymerase II parts. As a RNA binding protein, DGCR8 (Pasha in flies) recognizes the RNA structure. Drosha and DGCR8 form the microprocessor complex. While DGCR8 recognized the small RNA molecule , Drosha cuts the pri-miR and forms pre-miR structure.Pre-miR is carried from nucleus to cytoplasm by exportin-5 (exp-5). It is claimed that besides transporting of pre-miR, exp-5 protects the pre-miR from any destruction factor in the cytoplasm [37]. pre-miR is cut by RNaz III enzyme, Dicer. Dicer cleaves the round part of hairpin and forms 22 nt long double-stranded mature miR. Dicer recognises the double- stranded part of pre-miR and uniteds with protein. These proteins are not needed particularly and yet they enable the stabilization of Dicer-miR complex [38]. The strands of miR open and are divided into two as guide miR and passenger miR. Guide miR is more stabile than passenger miR at the 5'. Guide miR is binded the RNA-induced silencing complex (RISC) complex (TRGBP, PACT,Ago-2) Lately the imporatance of Ago-2 has been increased. Ago is a protein consisting of characteristic region like PAZ, mid. When guide miR is produced Ago is binded to miR from 3' and 5' . This complex suppress the protein expression. microRNAs form complex Binding to RISC cause ending translasion or destruction of target mRNA. miR is binded 3' untranslated region (UTR) , 3' open reading frame (ORF) or 5' UTR of target miR. This binding alters according to the bases arrays. mRNA is either destructed or protein remains untranslated by Ago2[45]According to bioinformatics results , there is a strong possibility that 3' UTR of genes might be a target of various miRs [46]. While in

UTR region there is non-complementary, in ORF region there is a perfect complementary. Besides this process miRs an also take control gene expressing directly Binding to target after maturazing without Binding RISC (Figure 10).

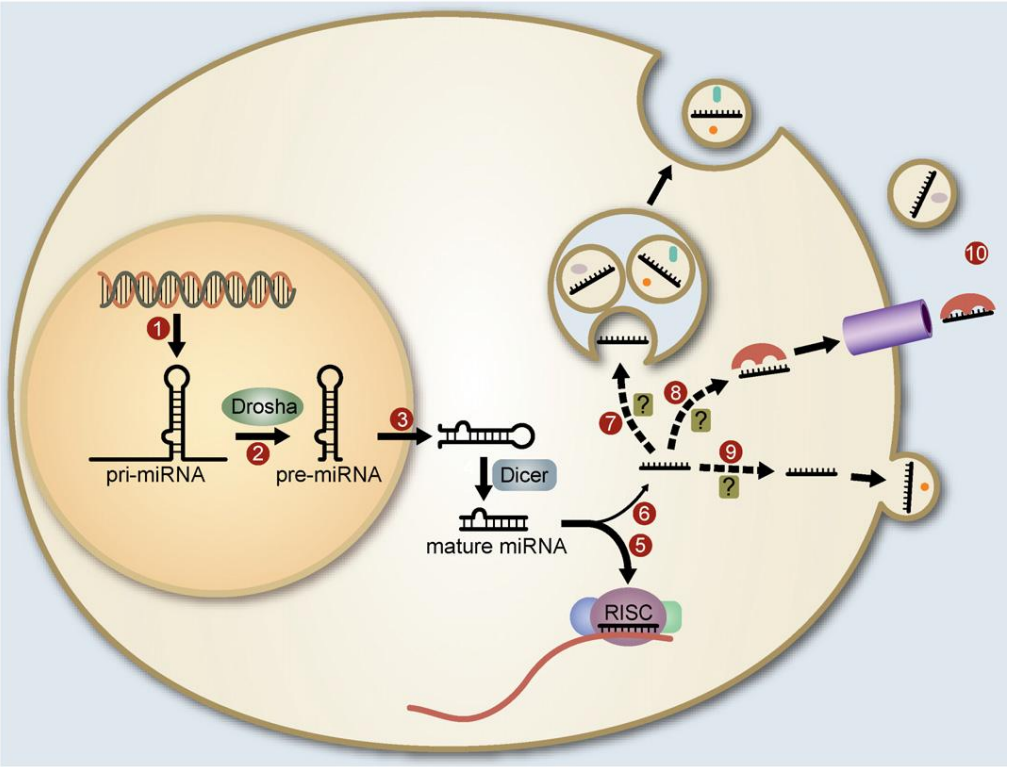

Figure 10: microRNA biogenesis process [39]

There are some differences between animal miR biogenesis and plant biogenesis. The main difference is in plants miRs are not exported from nucleus and unlike animal miR biogenesis pri-miR and pre-miR structures are formed by Dicerlike1 gene [40].

miRs in circulation system have some characteristic properties compared to the other miRs. Circulating miRs (CM) are resistant to chemicals and RNases also have serious stability . CMs present similarties with miRs in tissues so one can be claimed that CMs are avaible for being used as a biomarker.

4.3 microRNAs a biomarkers of diseases

Many studies have showed that miRs can be used as a diagnose and therapeuticpurposes. Also miRs have potential to predict treatment outcomes. Chronic lymphocytic leukemia (CLL) is the first evidence of miRs and cancer relationship. In 2002 Calin et. al. was proved that miRs had a important part in CLL. After studies it was stated that in CLL miR-15-a and miR-16-1 were down-regulated by analyzing the 13q14 gene [41]. miRs mostly place the least heterozygote regions in the genes, this allows the miRs damage the gene and cause specific cancer types. After Calin et. al study many scientists have focused to explain which miR an its expression cause which cancer type. Last studies are about breast cancer and mir21[42], non -small cell lung cancer and miR148-b [43]. In theory to cure the cancer antimiR or tumor suppressor miR is sent because hybridized miRs are not able to upregulated or down regulated [44]. Normally quite an amount of miR is not active in tumor cells. According to a study in 2012 by the World Health organization (WHO) the deadliest cancer types are lung cancer, liver cancer, stomach cancer, colorectal cancer, breast cancer, esophageal cancer <http://www.who.int/en, 27.09.2015>.

Table 2: miRs and their relationships with various diseases

miR	**Expression**	**Disease**	**Reference**
miR29a/b/c	Down-regulated	Lung cancer	[45]
miR218	Up-regulated	Liver cancer	[46]
miR139	Down-regulated	Gastric cancer	[47]
mir592	Up-regulated	Colorectal	[48]
miR21	Up-regulted	Breast	[49]
miR-3651	Down-regulated	Oesophageal cancer	[50]
miR-125a-5p	Down-regulated	Colon cancer	[51]
miR-19a	Up-regulated	Asthma	[52]
miR-494	Up-regulated	AIDS	[53]

4.4 miR based theraphy

Cancer can affect the miR biogenesis [54] so to clearify the miR expression it is important to explain all the steps and reasons of miR pathway. Because miRs have more than one target , there are opinions that miR theraphy is much more effective than siRNA therapy. But it has not been proved completely yet, there should be more optimization studies [55]. Besides cancer miRs also affects neurofunction of brain and neuron metabolism.

miRs affects paracrine signalling which is responsible for communication with neighbour cells. For example neurotransmitter communication between two close cells is a paracrine communication[56]. microRNAs play an important part in organ transplantation. It has been known that microRNAs regulate the cell communication and inflammation [57].

4.5 miR21

miR21 is considered as a oncogenic because in many cancer types , miR21 increases the uncontrolled cell division. miR21 is not related to the only breast cancer. Studies in time indicate that in in breast [49], colorectal [58], lung [59], gastric [60] cancers miR21 plays a vital role. Still mechanism of miR21 regulation has not been fully understood. PPARa and VHL is also declared as miR21's target [61]. It is observed that after medical treatment with antimiR21, vital resistance of Hepatocellular carcinoma (HCC) cell lines is descreased. Also this treatment triggers capsase activity which is related to the apoptosis and inhibits cancer cell growth [62]. There are studies that refer placenta has miRs in it but further researches are needed to be done [63, 64]. According to the PCR results miR21 in placenta is upregulated and related to the macrosomia [65]. miR21 is also related to the prostate cancer. It discreases the $TGBRF_2$ gene level and influences the epithel cells by targetting 3' UTR region [66].

5.CONDUCTING POLYMERS

5.1 Inroduction

Polymers are appriciable important field of chemistry. It has been developed since 20th century. Polymer word comes from Greek . Poly means many and meros

means parts. In other word polymers stands for macromolecules which are binded together with covalent bonds. Today high quality, inexpensive, effective materials are quite needed due to the rapid development of industry. One of important member of this material is polymers. Polymers have been used in wide range of fields from artificial organs [67, 68] to sensing technology [69-73] .

First CP was introduced by Shirakawa and collegues increased conductivity by dopping polyacethylene with oxidizing agent. In 1979 Diaz et al. oxidized pyrrole electrochemically and produced strong PPy film on anode. In 2000 they won the Nobel Prize. If CPs are compared to metals, CPs have benefits such as light, low cost, take form quite easily.

Usually polymers are insulators, or have very low electrical conductivity. It is because CP structure has long conjugated double chains. It is known polypyrrole, polyaniline, polytyophen are CPs, some of them are produced commercially as powder, suspantion, films, sheets.

To be conductive for a polymer some circumstances must be achieved.

1. Electrones are needed to be carried along the polymer chain to achieve this some proper places must be took part on the chain. Conjugated bonds provide this condition . Conjugated bonds progress single and double bonds.

2. Every conjugated bond is needed to have strong sigma bond.

3. Every double bond has pi bond which is less localized than sigma bonds.

CP films present both ionic and electronic conductivity. These films can be categorized in three different classes: Redox polymers, CPs, iox exchange polymers. Pyrrole is one of the redox polymers. Redox polymers conduct electricity by hopping from redox(oxidized and reduced) regions.

5.2 Conducting Polymers and Conductivity

CPs or conjugated polymers are defined as polymers binded to the main chain with weak electrones. In order to polymers present Conductivity, there must be approprite places providing electrones conducting..In conjugated bonds, carbons are binded as single and double with sigma bonds (σ). Additionally each bond has pi (π)

bond. When a monomer introduced dopant , specimen' s hole are increased. Holes enable the electrones transmit further away.It is called hopping.

5.2.1. Ionic conductivity

It has not been lighten up but there is one opinon is expected mostly: electrolite's anion or cation is binded to the polymer chain groups. As a result of this, the polymer starts to show changings of its shape, ions are moved from a group to the other. If a potential were to be applied ions are moved one direction.

Generally in ionic conductivity there is an electron giving group in polymers. These gropus make weak bonds with cation of the salt. Polimer solves ion or ions of the salt and makes easy for ions to seperate. These ions work as a charge carries. If anion or cation were to binded to any group on the polymer chain, they would be carried through the chain by bending as a result of heat.

5.2.2. Electrochemical conductivity

Band theory is one of the most acceptable theory for the electronic conductivity. Organic materials have high energy electrons and delocalized electrones with high band gap. Because of there reasons they are insulators. Conductivity occurs when electrones move freely. After giving away the energy to the organic monomers, the gap between two valance bands is closed and these monomers demonstrate a conductivity between metals and insulators. Electrones in the valance shell can be separeted and become positive charged or give one electrone to the empty conductivity band.

5.3 Polypyrrole

Pyrrole is a heterocycle with five members , aromatic monomer. Pyrrole's Chemical formula is C_4H_4NH. It is liquid at room temperature. Pyrrol is a liquid of which boiling point is 131^oC. It is affected by the light and dissolved in organic solvents and Py has $-NH_2$ groups on its chain. In addition Py is oxidized at +0.8 V[82].First pyrrole was obtained heating bones without any air and distilled as a result of this proccess red intense matter was produced , this res matter was distilled and pyrrole was produced for the first time.

Table 3 : Element in Polypyrrole [74]

Elemental Analysis of Polypyrrole			
%C	%H	%N	%Cl
48.70	5.60	13.90	8.70

Polypyrrole is one of the most popular concucting polymer used in biosensors [75]. As a conducting polymer polypyrrole is being more important due to its advantegeous properties such as biocompability, being able to study in neutral pH, pH ranges from 3 to 9, high electrical conductivity, easy to synthesis, good enviromental stability, fast electrochemical response, inexpensive, high surface energy [76-80]. PPy cannot be melted because it has weak interactions with electrolyte and increases the stability of sample. Especially pH tolerence of PPy enables the studying living cells, biomaterials also protects the electrode from contamination [75]. Conductivity of PPy ranges from 10^{-3} to 10^{3} Ω/cm [81].

The story of pyrrole begins in 1916, Angeli et. al oxidized the pyrrole and got a black powder. At first it was called pyrrole black but this is not conductive because it contains so many oxygen. First electropolymerization of Py was by Diaz et. al. in 1979. Diaz oxidized pyrrole Electrochemically and produced onto anode and its conductivty was 100 S/ cm. During the polymerization, pyrrole gives precipitate and polypyrrole structure depends on electrolyte, pyrrole concentration, pH. Livache, T., et al. mentioned that PPy is coated to the electrode surface only.

PPy is used in many applications such as controlled drug releasing [82], gas sensors, biosensors, chemical sensors, immunosensors, artificial muscles, nanorobots [83], batteries, super capasitors, solar cells, field- effect transistors, enery storage devices [84], corrosion conservation .

5.4 Polypyrrole Synthesis

PPy can be synthesized various ways. Chemical and Electrochemical methods are the most used.

5.4.1 Chemical synthesis of polypyrrole

In this method pyyrole is introduced the oxidative agents or catalyts and they affect the conductivity. Although it has some advantages but it allows to deposit PPy to the electrode surface with poor adhesion and unable to control the oxidation process compared to Electrochemical syhthesized PPy deposition on the electrode surface. Also the product is not all pure.

5.4.2 Electrochemical synthesis of polypyrrole

Electrochemical polymerization (Figure 11) has many benefits. Electrochemical polymerization allows the controlled PPy film thickness and mophology [75], studying in wide range of pH is possible, entrapment of biomolecules can be performed successfully. Controlling these conditions and depositing polypyrrole on the electrode surface can de achieved while polymerization of pyrrole proccess [85]. Therefore CPs behave stable [86]. Reactions occur at the room temperature, polymer film is obtained on the electrode surface, it is possible to dope any ion at the same time electropolymerization. CPs can be used in order to discriminate between mismatch sequence and complementary sequence successfully [87].In this methot monomer is put into electrochemical cell with appropiate electrolite. By applying the right potential polymerization easily succeed. Mostly CV is used. Mostly constant current or potential is used. Electropolymerization or deposition electrochemically means that a monomer is oxidized anodically and gathered on the electrode surface. By electropolimerization, a quite stabil film is formed. Mostly aromatic ring structures form a conductive film. During electropolimerization aromatic ring is preserved. Reaction moves on. Electrochemical polymerization starts oxidizing monomer in the electrolyte solution. Electrolyte solution differs accoding to the purpose of the study. By using salt or dopant the conductivity might be increased. Because pi and sigma bonds are not enough to make polymer a conducting polymer. Refraing this reagents might harm the polymerization of PPy [88]. Electropolymerization can occur in two ways either monomer and radical cation form dimer or two radical cations form dimer.

Electropolymerization of PPy has 4 basic steps:

1. Monomer is oxidized in the electrolyte solution by giving electrone. Oxidized monomer forms radical cation.
2. Radical cation unites monomer and forms dimer. Dimers are also radical cations.
3. Radical dimer cation unites monomer and forms trimer.
4. Trimers unites monomer and this proccess keep on until polymer chain forms.

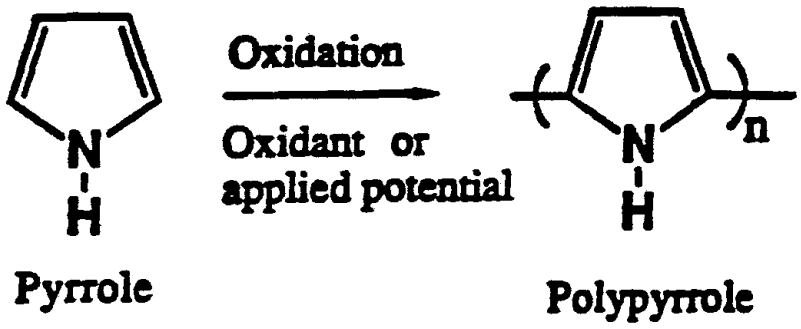

Figure 11: Pyrrole electropolymerization mechanism [89]

Electropolymerization of PPy is based on controlling factors like pH, temperature, working electrode,solvents, electrolytes. The most appropriate temparature for electropolymerization is between 10^{o}C and 30^{o}C [90].

During the polymerization of pyrrole, many electrochemical techniques such as voltammetric, amperometric, galvanostatic have been used for the deposition of PPy onto the electrode surface. One of the significant factor in the syhthesis of polypyrrole is choosing the WE. The electrode should not be oxidized during the polymerization and deposition. That's why mostly platinum [91] or gold electrodes [92]. Also stainless steel [93], iron [94], aliminum [95], zinc [96], titanium [97],pencil graphite electrode [98, 99], graphite rode electrode [90], screen printed carbon electrode [100] have been used. Metals like iron, silver is not used so much as an electrode because they are oxidized between polymerization potential ranges. Although gold, platinium are vey stable during polymerization but they are expensive becuse of these reason in this study pencil graphite electrode (PGE) was used. In addition PGE has other benefits. Such as low cost, desposible, rich Electrochemical reactivity, easy to prepare, no need for polishing [101]. But if this

two polimerizaiton techniques were to be compared, electropolymerization is at the advantage. Because in chemical polymerization needs expensive chemicals, long time for the process [102].

5.5 Polypyrrole and Biosensors

Polypyrrole has been studied in biosensors extensively [76, 103, 104]. To the best our knowledge DNA could be immobilized onto PPy matrix easily [105]. In DNA biosensors signal differences of interaction between probe, target and hybrid are important but in some detection methods, these differences are quite small. In order to amplify hybrid signal, conductive polymers are one of the significant canditate. Because peak height of the sample is related to the data transmitted from electrode. and CPs allow the datas transmit more easily due to their conjugate bond structure. In addition it is claimed that PPy is like a p-type semiconductor [106].

While designing a DNA sensor, it is important to choose the polymer. Because the polymer is needed to be compatible with the biomelocule used . The polymer creates an interface between electrode and biomolecule. PPy is one of the suitable polymer because PPy connects biomolecule and electrode more strongly and allows the electrons flow more efficiently [107]. Also before immobilization of biomolecules some factors should be considered such as good adhesion of surface, biocompitable polymer, thickness of the polymer film, squence of DNA or RNA [9].

Fabricating a biosensor using polymer requires 3 basic steps:

1. Immobilization of polymer

2.Immobilization of biomolecule

3. Suitable detection method.

5.6 DNA immobilization methods onto polypyrrole

Immobilization of biomolecules on PPy coated electrode basicly occurs in 3 ways:

5.6.1 Adsorption of DNA

DNA could be immobilized onto electrode coated with chemically [108] and electrochemically. Adsoption is of one most used immobilization method. Yet in adsorption, there are weak bonds between polymer matrix and biomolecule which could cause DNA seperating from polymer easily and also requires long time like 2.5 hours .

5.6.2 Entrapment of DNA

Py solution and the biomolecule are in the same electrochemical cell during the polymarization. With this both polymerization of Py and deposition of PPy and biomolecules are achieved [109]. As it is shown in the Figure12 that the Py solution and oligonucleotides are put together same and oxidized at the same time. As a result of polymerization, PPy has holes. These holes are filled by ions in the solution. These ions change places with DNA. Positively charged PPy, attracks the negatively charged DNA. The entrapped DNA acts as a dopant. PPy's negative charged dopant or ions alters places with DNA [110]. DNA is immobilized with Py during electropolymerization [88]. In addition DNA does not lose its hybridization ability [111]. This is because electrode coated with PPy and DNA has thin, porous surface, in addition these porouses enable the hybridization .In entrapment it is important to choose right parameters criterions such as potential,scan rate [112]. The entrapped probe is stabile and strong against enviromental factors like temperature, electrode's surface roughness.

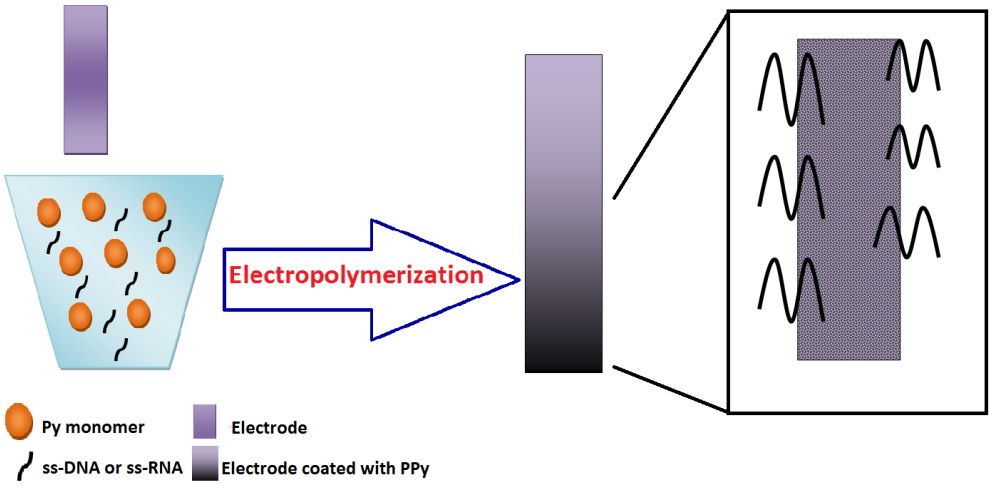

Figure 12: Entrapment of DNA

5.6.3 Covalent binding of DNA

Minehan et. al. stated that DNA Adsorption onto electrode coated PPy is diffusion controlled. If an covalently immobilization of DNA were to be claimed successful, there should not be any non-specific bondings. That's why immobilization step is really important. These non-specific bondings effects the outcoming results therefore the sensivity and reliability descreases [113].

6.MATERIAL AND METHODS

6.1 Apparatus and Chemicals

AUTOLAB PGSTAT204 Compact and modular potentiostat/galvanostat is used for application of CV and EIS techniques. Conventional three electrode system, consisting of the PGE as the working electrode, (Ag/AgCl) as a reference electrode and a platinum wire as an counter electrode are engaged in connection with the Nova 1.10 software.

All synthetic oligonucleotides listed are purchased from Alpha DNA (Canada).

Meldola's Blue studies are done in TBS (20 m M, pH 7.0) and EIS measurement were performed in redox probe of 0.05 M $K_3Fe(CN)_6$ solution.
Other Chemicals are obtained either from Sigma or Merck. All stock solutions are prepared Using ultrapure water.

Table4 : Sequences of microRNAs

miR21 (DNA)	5′-TAGCTTATCAGACTGATGTTGA-3′
antimiR21 (DNA)	5′-TCAACATCAGTCTGATAAGCTA-3′
miR21 (RNA)	5′-UAGCUUAUCAGACUGAUGUUGA-3'
antimiR21 (RNA)	5′-UCAACAUCAGUCUGAUAAGCUA-3'
miR122(RNA)	5'-UGGAGUGUGACAAUGGUGUUUG-3'
miR21-biotin	5′-TAGCTTGATAGACGGATATTGA-3I′

6.2 Methods

6.2.1 Preparation of buffer solutions

6.2.1.1 Phosphate buffer solution(PBS)

50 mM Phosphate buffer solution contains 10 mM potassium dihydrogen phosphate (KH2PO4) , 40 mM potassium hydrogen phosphate (K2HPO4) . After preparing the rough solution pH is arranged for the 7.40, finally 20 mm male sodium chloride (NaCl) is added.

6.2.1.2 Acetate buffer solution (ABS)

50 mM acetate buffer solution is prepared with concentrated acid. pH is arranged to the 4.80 by adding 1 M Sodium hydroxide (NaOH) and 20 mM NaCl.

6.2.1.3 Tris hydrochloride buffer solution (TBS)

20 mM Tris HCl soltion contains 0.2 mol Trizma hyrochloride and 20 mM NaCl. pH is arranged for the 7.0.

6.2.2 Preparation of miRNA stock solutions

The stock solutions of RNA oligonucleotides (1000 μg/mL) are prepared in RNase-free water daily and are stored at -20 °C ready to be used.

6.2.2.1 Total RNA isolation from cell lines

Total RNAs were obtained from MCF-7 and HUH-7 cell lysates. The isolation procedure could be found the following study [114]. MCF-7 is rich for miR21 which is target of this study. In order to prove antimiR21 is specific to the up-regulated miR21, HUH-7 was used. HUH-7 contains a large amount of miR122 and poor for miR21.

6.2.3Activating the PGEs

The bare PGEs are activated in the ABS (with 20 mM NaCl, pH:4.8) under the +1.40 V for 30 s and oxidized from +0.6 V to 1.45 V.

6.2.4 Electropolymerization and deposition of polypyrrole

Pyrrole solution (10 mM, 30 mM KCl, ABS pH:4.80) was polymerized using by CV with 5 cycles. Each cycle started at 0.0 V and ended at +0.85 V. Scan rate was 0.025 V/s.

6.2.5 Immobilization of probe/ entrapment of antimiR21

AntimiR21 was immobilized/entrapped using CV consisting of 5 identical cycles scanning from 0.0 V to +0.85 V with 0.025 V/s scan rate.

6.2.6 Solid phase hybridization of miR on PGE

PGE/PPy-antimiR21 was dipped into 100 µL target stock solution. It was waited for 30 minutes.

6.2.7 Hybridization of mir21 using cell lysates

The target cell lysates were isoleted from MCF-7 and non-complimentrary RNA were isolated from HUH-7 .The isolation procedure can be foun in following study[129] . MCF- 7 cell lines are rich in point of miR21 and HUH-7 cell lysates are rich in miR122. Total RNAs were diluted using PBS(pH:7.40) in order to get final concentration. Antimir21 were entrapped onto PGE/PPy electrodes Using cyclic voltammetry. After entrapment the electrodes were rinsed dipping once into PBS (pH:7.40).PGE/PPy-antimiR21 electrodes were put into PCR tubes containing 100µL target RNA solution either MCF-7 or HUH-7. The electrodes were preserved for 30 minutes , after that the electrodes were rinsed by dipping once into PBS (pH:7.40).

6.2.7 Intercalation of Meldola's blue

Meldola's blue(3 mL, 20 µg/mL)solution was deposited under +0.5 V for 300 seconds with stirring.

6.2.9 EIS measurements

The EIS studies were achieved in the 5 mM K3Fe(CN)6 solution . Using +0.24 V AC voltage and 60 logarithmical points , From 10-1 Hz to 105 Hz frequency were used Using 60 logatihmical points. The information of electrode surface was studied Calculation Rct values of semicircle.

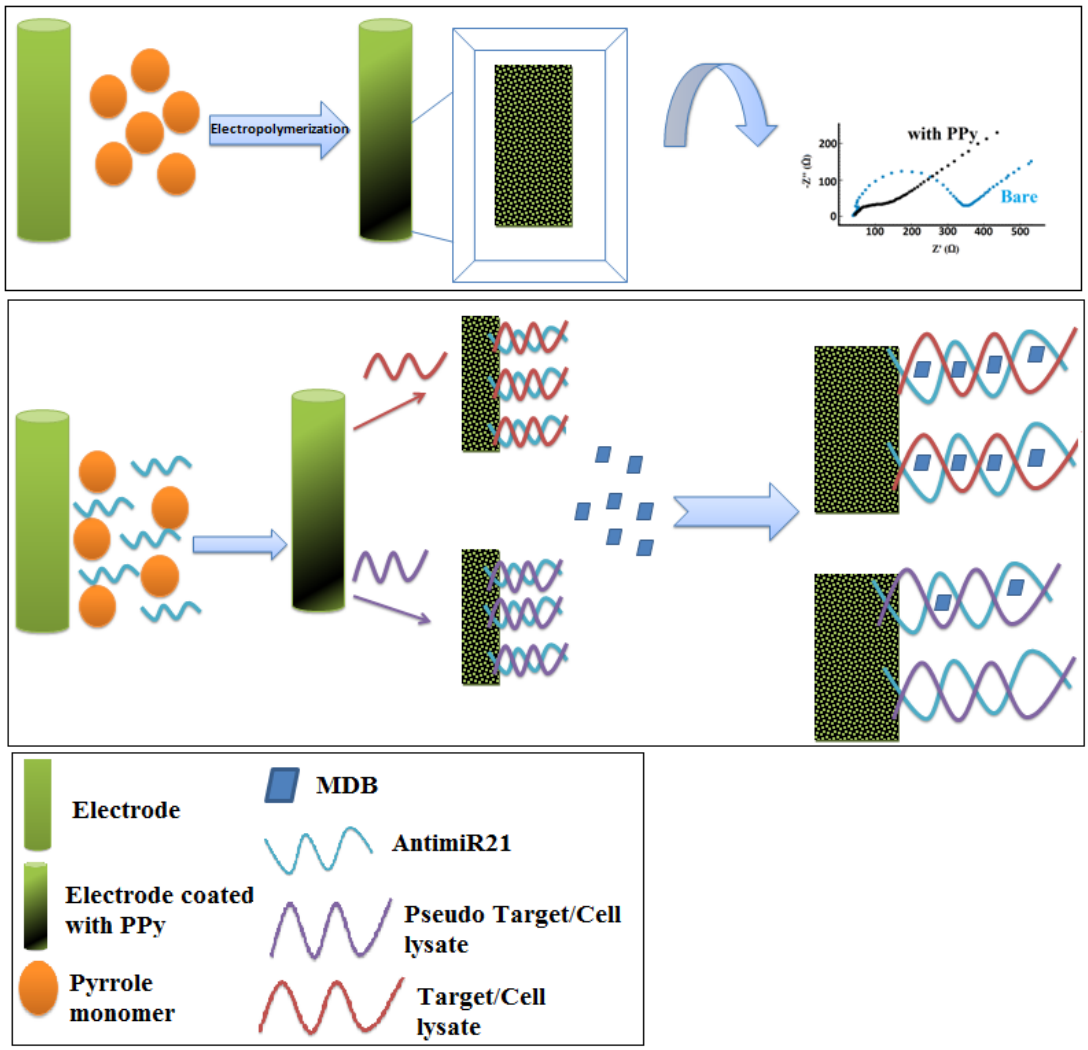

Figure 13 : Schematic presentation of the study

7.RESULTS AND DISCUSSIONS

Results and discussions section contains optimization studies, detection with synthethic miRNAs and detection inside total RNAs.

7.1 Optimization Studies

Regarding optimization studies, cycle repetition in electropolymerization of PPy, pH calibration, target concentration study, Hybridization time Optimization were performed.

7.1.1 Cycle Repetition in electropolymerization of polypyrrole

In electrochemisty surface morphology has an important role with regards to better results. The thickness of the electrode surface affects not only the surface

morhology but olso immobilization of biomolecules [88]. The case is similar when CPs are studied ; if the polymer film is really thick, the conductivity of electrode descreases and electrode beheaves like an insulator due to degradition [77].

CV is one of the most used methods for polymerization. If CV is used for an electropolymerization with more than one cycle, it would be wise to be careful while selecting the cycle number. The cycle number affects the thickness of the electrode surface.Therefore the conductiviy is affected. After too many cycles the thickness is increased and current flow descreases. The cojugated bonds are broken and conductivity descreases [115]. In addition immobilization of biomolecules like DNA is not achieved [116].

Due to reasons above 5 cycles were chosen for the electropolymerization. The polimerization started at 2 cycles and stabilizes between 4 and 6 cycles. There is a slight increase of Rct value after 6 cycles. In order to carry out a succesful polymerization 5 cycles were used. Because 5 cycles are in the stabil zone of the Figure14.

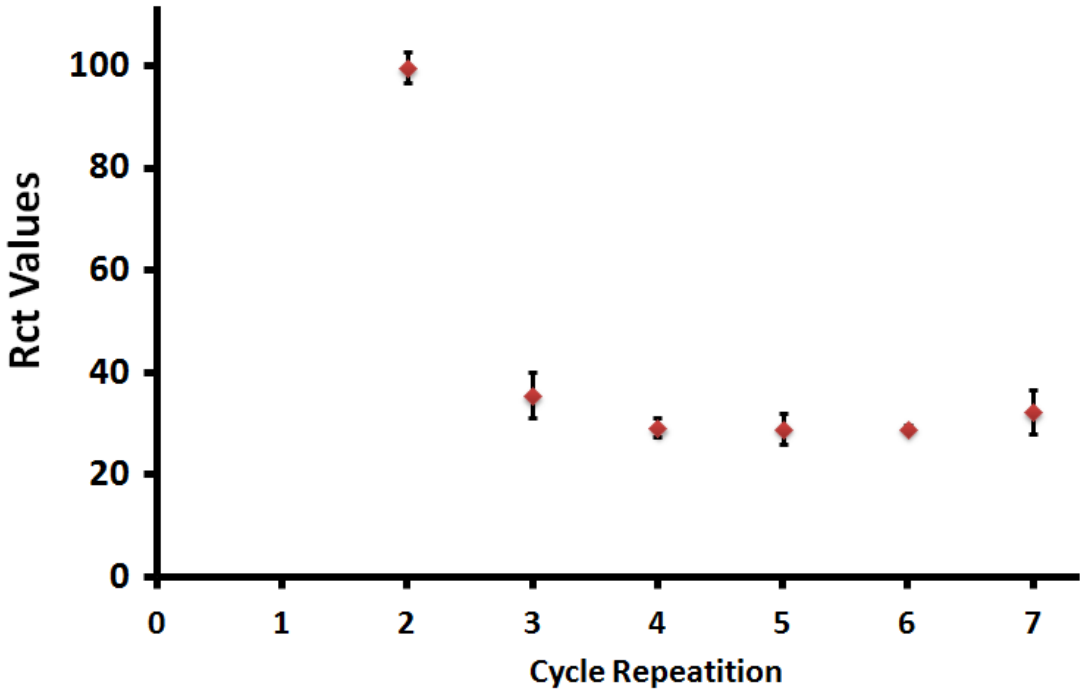

Figure 14: Optimization of the cycle Repetition in electropolymerization of PPy from 2 cycles to 7 cycles.

7.1.2 Optimization of pH in electropolymerization of polypyrrole

The study rage is claimed as pH 3-9 . In previous studies it is indicated that polymer films were more stabile at low pH [117]. Especially at pH: 1 and pH: 7 the condutiviy is fairly inadequate. In addition if the potential more between +0.8 V and +0.9 V is applied, the PPy signals are observed more specifically[118]

In this study there is another factor that needs to be considered: microRNA. DNA is active between pH: 3 and pH: 8 and has the best immobilization between pH: 4 and pH: 5. In addition RNAs display the most stabile behaviour between pH: 4 and pH: 5 [119] and unstabile behaviours at alkaline pHs [120]. In addition pHs more than 8 both DNA and RNA starts to denature and at pH lower than 3 not only the phosphadiester bonds but also the hybrogen bonds between bases are broken. Also it is claimed that RNA is fit for acidic enviroment [121]. Besides it is easy to immobilized microRNA into positively charged PPy in acidic enviroment [117].

Py solution was prepared using ABS(pH: 4.80) for electropolymerization regarding reasons above. In the figure15 , it is observed that high conductivty and immobilization are between pH: 4 and pH: 5. With this conductivity of electrode was increased and antimiR21 was immobilized onto electrode surface firmly.

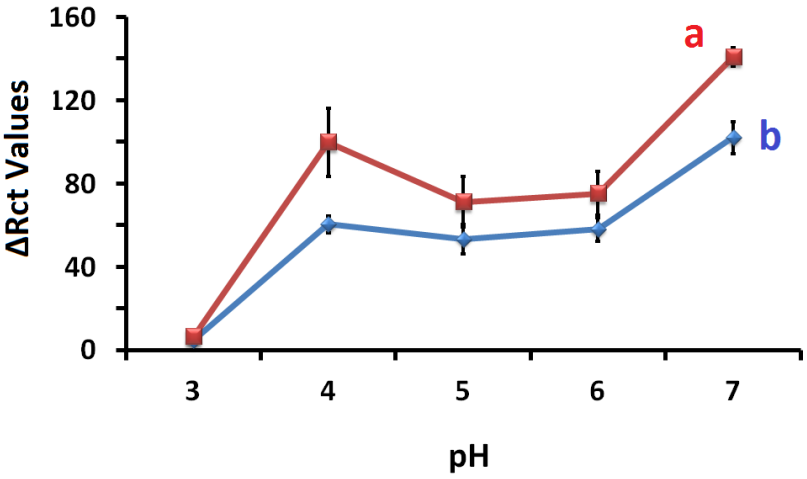

Figure 15 : Optimization of pH lineer graphic a) probe immobilization study depending on optimization of type of immobilization of miR on ppy coated electrode different at pH: 3,4,5,6,7 of pyrrole solution b) pH study of pyrrole solution at pH: 3,4,5,6,7

7.1.3 Comparing physical adsorption and entrapment

Immobilization is one of important factor for a succesful biosensor. Immobilization could be performed in many different ways. Entrapment is one of these ways . On the other side there is one of the most used immobilization method adsorption. It is shown in Figure16, the entrapment demonstrated more Rct values than adsortion did. The results indicated that entrapment is more suitable immobilization method for the this study. Due to strong attractions between them antimiR21 was immobilized more succesfully in entrapment. The entrapment process was explained in detail in section 5.6.2. Also PPy film has a porous structure. The antimiR21 is immobilized to the electrode surface thoughy the porouses and the hybridization does not affected [117].

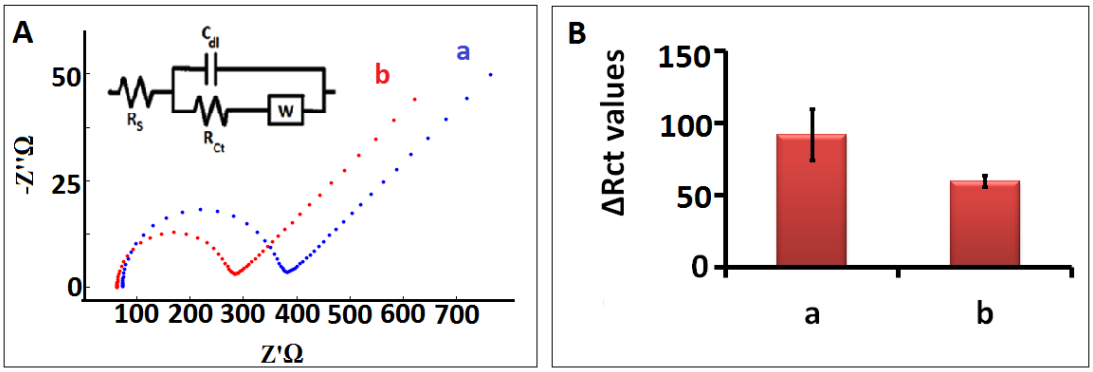

Figure 16 : Comparation of phsical Adsorption and entrapment of antimiR21 onto electrode with Nyquist diagrams(A) and column graphics with error bars (B). a) entrapment of antimiR21 onto electrode surface with PPy b) phsical Adsorption of antimiR21 onto electode coated with PPy.

7.1.4 Concentration study of antimir21 immobilized on working electrode

Immobilization is a signaficant factor in designing a biosensor.Yet choosing method is not enough alone. The determination of the probe concentration is another fact. The probe concentration right before the saturation give information about the electrode capacity for the certain probe .

In figure 17 it is easily observed that The Rct values increases till 5 µg/ml after that there is a descrease of Rct values till 7 µg/ml and stability at 10µg/ml.Because after 5 µg/ml the saturation begins. It could be concluded that the most suitable probe concentration of this study is 5 µg/ml.

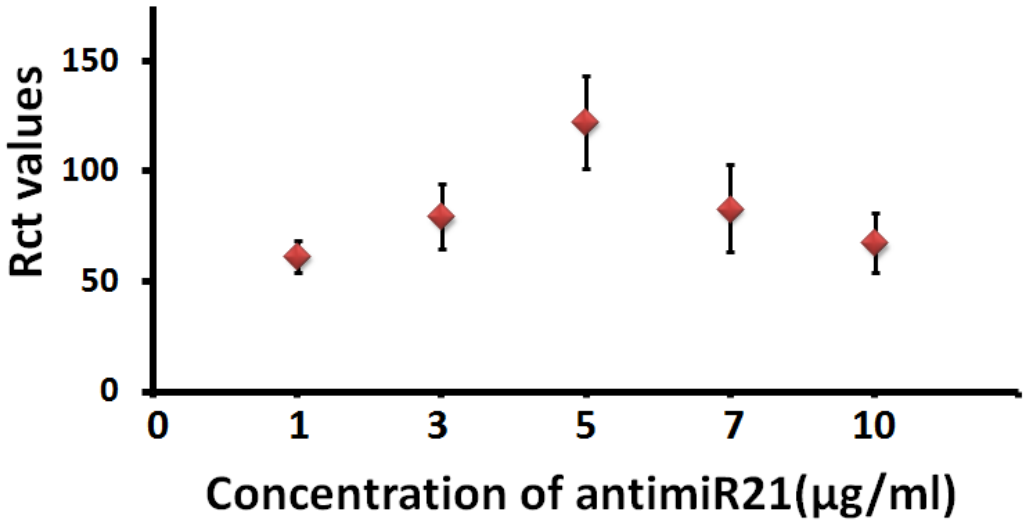

Figure17: Optimization of antimiR21 upon concentration increasingly from 1 µg/ml to 10 µg/ml

7.1.5 Optimization of hybridization time

Finding the right hybridization time for biosensor plays an essential role because it affects of the biosensor selectivity as much as the other parameters like prob/target concentration . Also in order to get lower LOD it is mentioned that Hybridization time needs to be long [122]. In this study Hybridization time was varied from 10 minutes to 50 minutes. The MDB reduction signal increased till the 30 minutes and after that it started to decrease till 40 minutes and finally it saturated at 40 minutes. Even at 50 minutes the response didn't change.

Figure 18 reveals that the optimum time for Hybridization proccess is 30 minutes. Because of MDB intercalated between double helix and in full match and successfully Hybridization it reveals the maximum reduction signal.

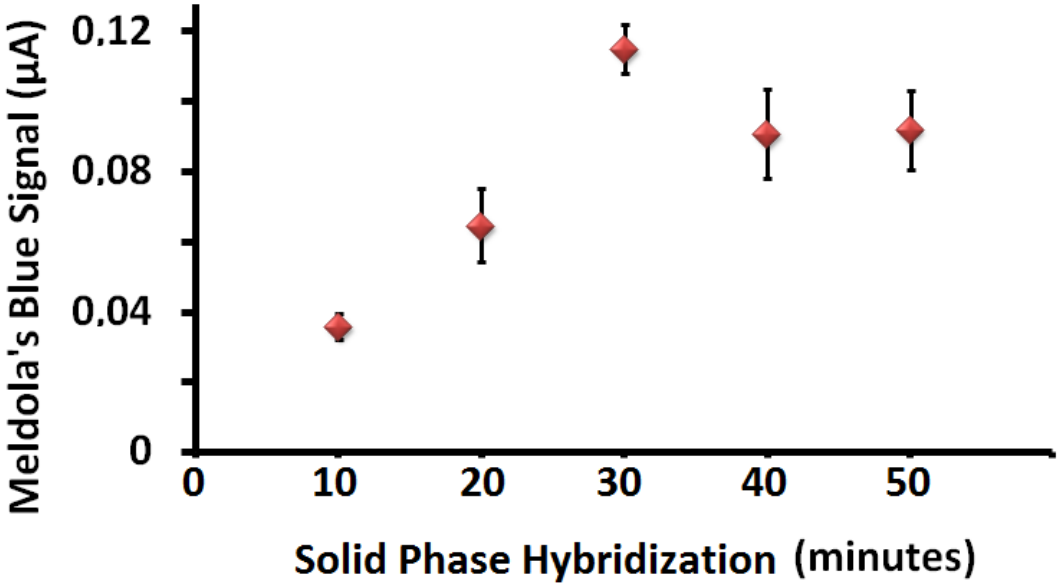

Figure 18 : The optimization of hybridization time ,while probe and target concentrations weren't changed, hybridization time was varied. First hybridization time was selected as 10 minutes.

7.1.6 Concentration study of target

Figure15 reveals the impact of the target concentration onto the Hybridization signal. the probe concentration (5 µg/ml) was kept still and target concentrations were varied from 1µg/ml to 10 µg/ml. The MDB signal increased to the 7 µg/ml linearly and started to descrease which means that there is saturation [122, 123]. The highest MDB reduction signal was obtained from 7 µg/ml of target. Due to reasons before explained 7 µg/ml was used as target concentration of miR21(Figure 19).

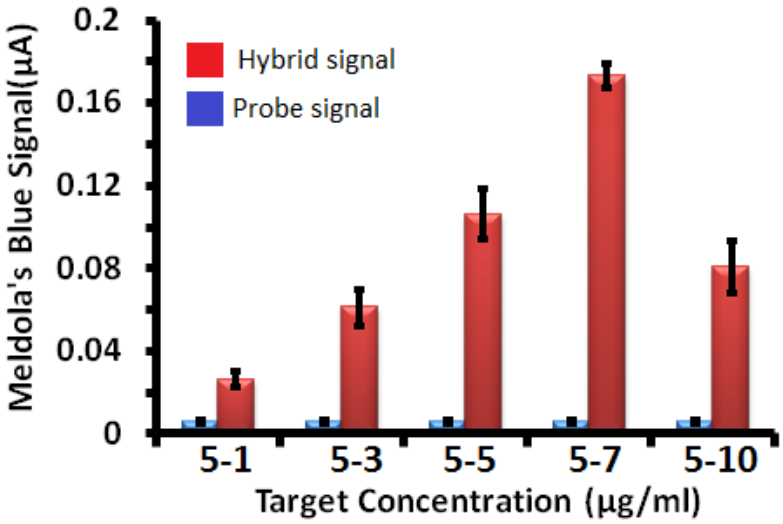

Figure19: Target concentration column graphic based on MDB signal agains to varied miR21 (target) concentrations while probe concentration (antimir21, 5µg/ml) . From 1µg/ml to 10 µg/ml the target concentration were changed. Blue columns represent the probe and red columns represent the target MDB signal due to concentation changes

7.2 Electrochemical Detection of miR21 on Polypyrrole Coated Electrodes

After all optimization steps were completed, the electrochemical detection of miR21 by PPy coated PGE was achieved. As the control studies the non complementary target, mismatch target were used. Hybridization were detected by using EIS and DPV.

Figure 20 exibits the nature of the electrode hybridization coated with PPy. Due conjugated bonds on the PPy chain , electrones are conductted faster. This makes using PPy so feasible. It is showed that EIS, Rct values can be used in order to monitor hybridization[33]. The non complemetary hybrid (PGE/PPy-antimiR21/miR122) exibitted almost the same response as the probe(c). It is due to unmatched base pairs of antimiR21 and miR122. Fig. B exibits the hyridization of probe without using PPy . antimiR21(PGE/antimiR21,y) was immobilized onto the bare PGE(x), and hybridized with miR21 (PGE/antimiR21/miR21, t) and noncomplementary target, miR122 (PGE/antimiR21/miR122, z). It is clearly observed that after using PPy the Rct values descreased . Section C shows the the Rct differences between bare PGE and PGE/PPy electrodes. It is found that the PGE

coated with PPy shows 4 times more conductivty than bare PGE. The rise of Rct values is due to the negativty of antimiR21 and $K_3[Fe(CN)_6]$ [124].

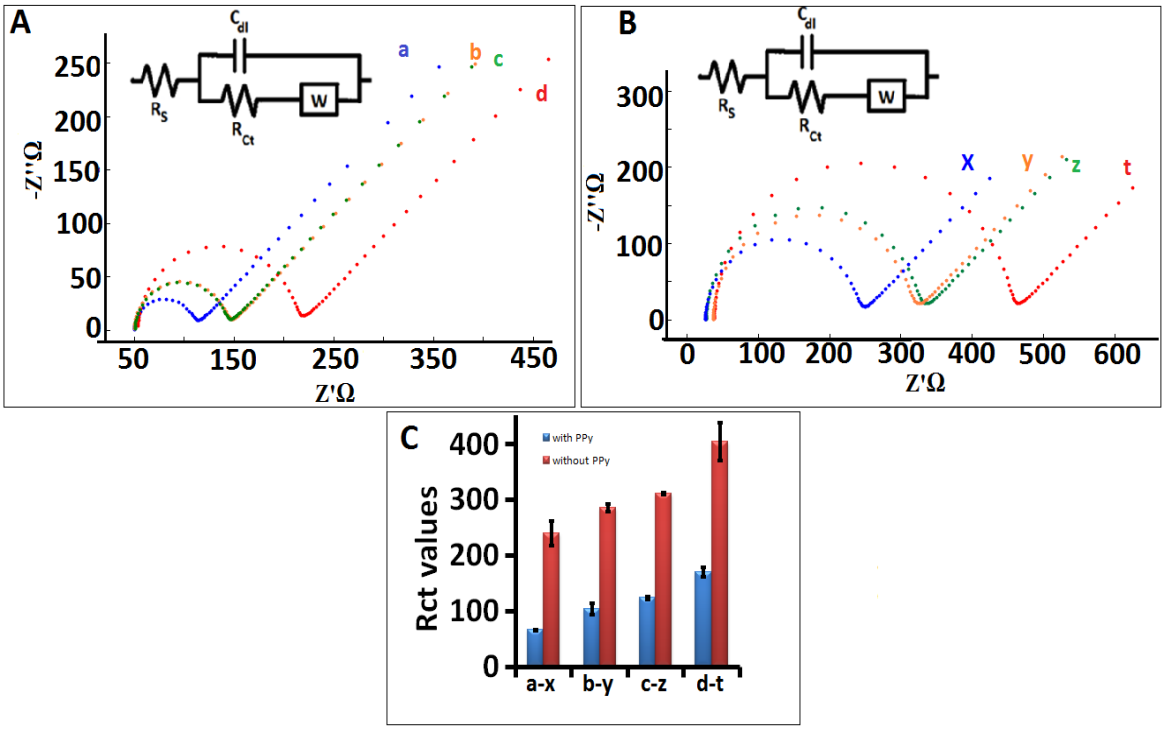

Figure 20: Results of the Hybridization study via Nyquist plot diameter . A PGE coated with PPy (PGE/PPy,a), entrapped antimiR21 onto electrode surface with PPy matrix (PGE/PPy-antimiR, b), after Hybridization (PGE/PPy-antimiR21/miR21,d), after non complementary imoobilization onto probe (PGE/PPY-antimiR21/miR122). B Bare PGE(PGE, x), antimiR21 immobilized PGE (PGE/antimiR21,y), hybrid (PGE/antimiR21/miR21,t), after non complementary imoobilization onto probe(PGE/antimiR21/miR122,z)

Figure 21 exibits DPV voltammograms and column graphics with error bars of determination study of the hybridization using MDB reduction response. Due to nature of the MDB it is expected that the highest peak to be belong to the hybrid. There are some MDB signals at PGE coated with PPy (PGE/PPy,a), antimiR21 entrapped PGE (PGE/PPy-antimiR21,c) and The non complemetary hybrid (PGE/PPy-antimiR21/miR122 , b). While immoblizing MDB a potential was applied and it is natural to bind MDB to the other samples. In addition there are many studies in the literature that show the bare, probe have really low MDB signals compared to the hybrid [122].

The lowest response was noted at PGE was coated with PPy. The MDB signal increased when antimiR21 was entrapped onto the PGE surface using PPy as a linking agent. After entrappment, antimiR21 was hybridized with miR21(PGE/PPY-antimiR21/miR21,a) and exibited the highest peak. the non complemantary hybrid(PGE/PPy-antimiR21/miR122,b)exibitted almost the same response as the probe(c). It is because antimiR21 and miR122 have no common base pair .Peaks were observed at nearly -0.17 V which are suppoted by the studies [28]. After multiple repeats of the study the standard deviation of the samples were calculated and found really identical. Each sample presents pretty good internal consistency . This conclude is obtained from the standard deviation values and error bars.

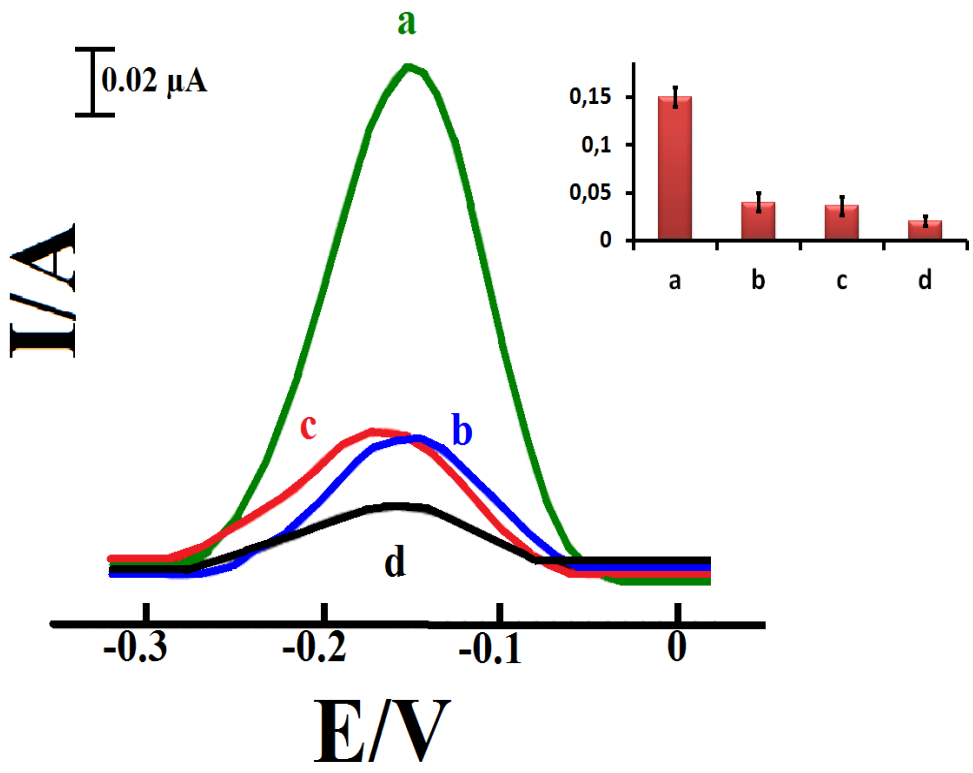

Figure 21: MDB signal voltammograms and column graphics with error bars for antimiR21(RNA) Hybridization. a)PGE/PPy-antimiR21/miR21(RNA), b)PGE/PPy-antimiR21/miR122(RNA), c) PGE/PPy-antimiR21,d) PGE/PPy vs Ag/AgCl

Figure 22 presents DPV voltammograms and column graphics with error bars ofbelong to the determination of mismatch hybrid using MDB reduction response. The response of MDB was monitored at nearly -0.17V.The lowest MDB

signal belongs the PGE coated with PPy (PGE/PPy,d). The MDB signal increased when antimiR21 was entrapped onto the PGE surface with PPy matrix (PGE/PPy-antimiR2,c). Full mstched hybrid (PGE/PPY-antimiR21/miR21, a) presents the greatest MDB response. The mismatch (PGE/PPy-antimiR21/miR21 , b) exibitted a little higher response than the probe(c). The mismatch has both paired and unpaired bases because of the MDB' s intercalating nature ,it is predicted to have this results.

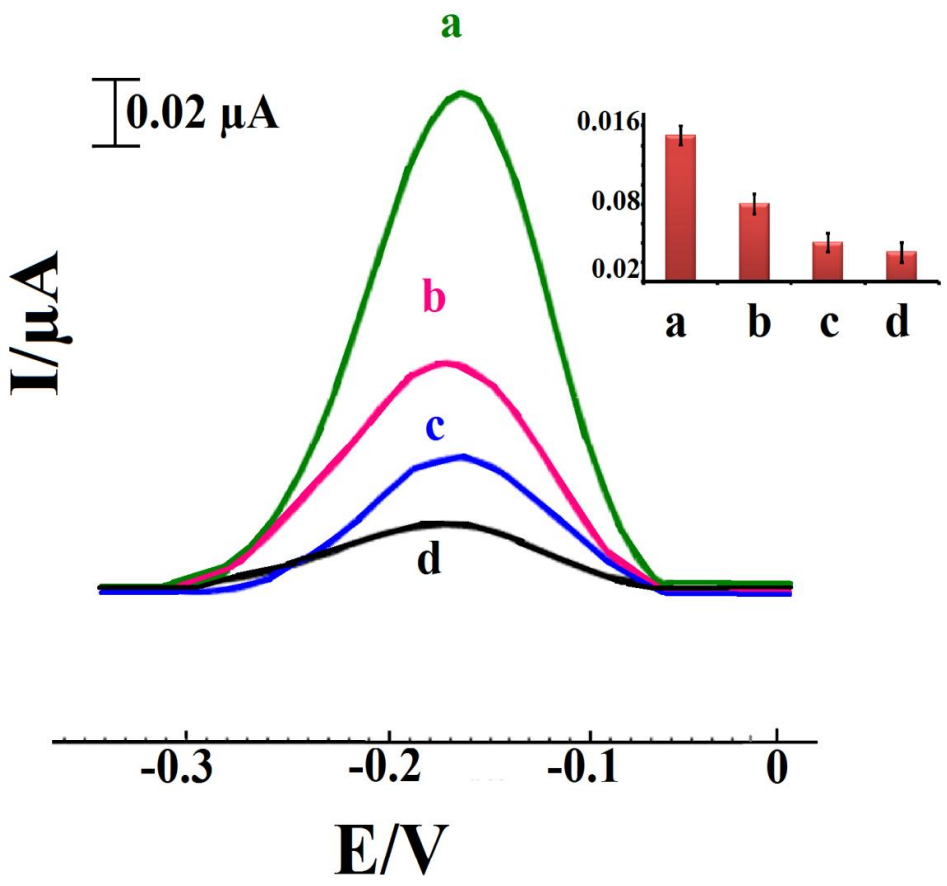

Figure 22: MDB signal of mismatch study voltammograms and column graphics. a)PGE/PPy-antimiR21/miR21(RNA), b)PGE/PPy-antimiR21/miR21(), c) PGE/PPy-antimiR21,d) PGE/PPy vs Ag/AgCl

As it can be clealy seen in the Figure 23, breast cancer biomarker miR21 was detected in total RNA easily . In order to be sure that antimiR21 is only specific to miR21, cell lysates of total RNA(MCF-7, HUH-7) were used. MCF-7 is rich for miR21. HUH-7 is rich for miR122. antimiR21 and miR122 have no mutual base

pairs. It is expected to have no hybridization signal from HUH-7 [26]. Yet cell lysates do not contain only one type of miR but also impurities, the other miRs but a miserable amount. That is why well defined peaks are not observed. Due to Electrochemical adsoption PGE/PPy(d) also showed a slight MDB reduction signal. Due to same reason as PGE/PPy , PGE/PPy-antimiR21(c) presents a MDB peak but higher response compared to PGE/PPy. The highest peak is observed at PGE/PPy-antimiR21/MCF-7(a). The noncomplementry hybrid(b) presents the almost the same response as PGE/PPy-antimiR21. Like antimiR21 (RNA) studies, the MDB reduction peaks were observed at -0.17V. From the total RNA results it is conclueded that antimiR21 is specific to miR21 is not hybridized in any other samples that are lack of miR21.

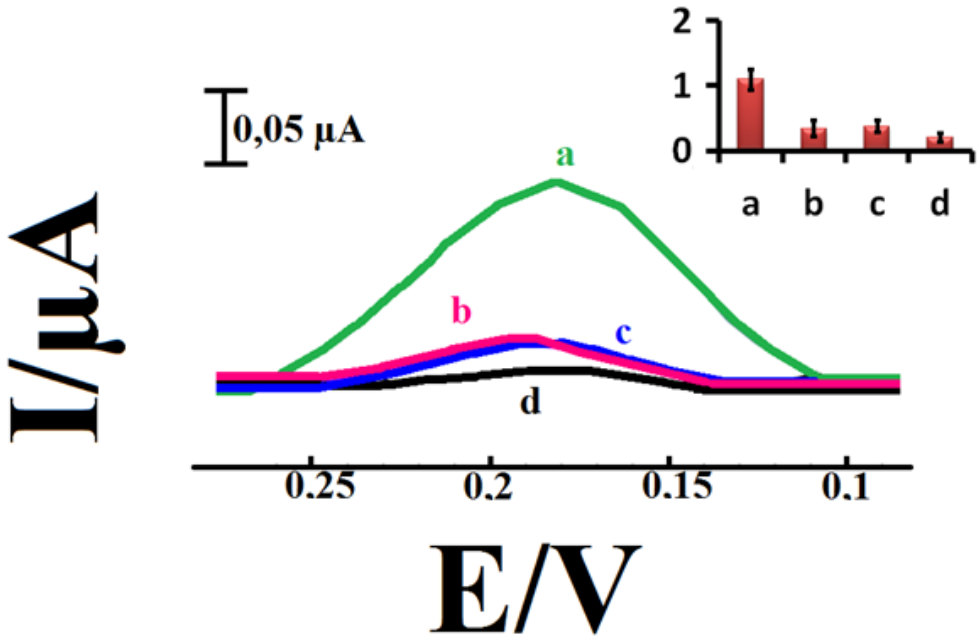

Figure 23: MDB signal voltammograms and column graphics for MCF-7 cell lysates hybridization. a)PGE/PPy-antimiR21/MCF-7, b)PGE/PPy-antimiR21/HUH-7, c) PGE/PPy-antimiR21,d) blank vs Ag/AgCl

Figure 24 exibits the nature of the electrode hybridization coated with PPy. After immobilizing the antimiR21 onto PGE using PPy matrix, R_{ct} values increase compared to PGE/PPy. The hybrid is expected to have the highest R_{ct} values. Because R_{ct} is the resistance of polarization. Depending on the polarization Rct values are changed. For that reason when a biomolecule is introduced to $K_3[Fe(CN)_6]$, due to repulsion of the negative charges, the Rct value increases On the

other hand . The pseudohybrid (PGE/PPy-antimiR21/HUH-7) exibitted almost the same response as the probe(c) due to uncommon base pairs of miR122.

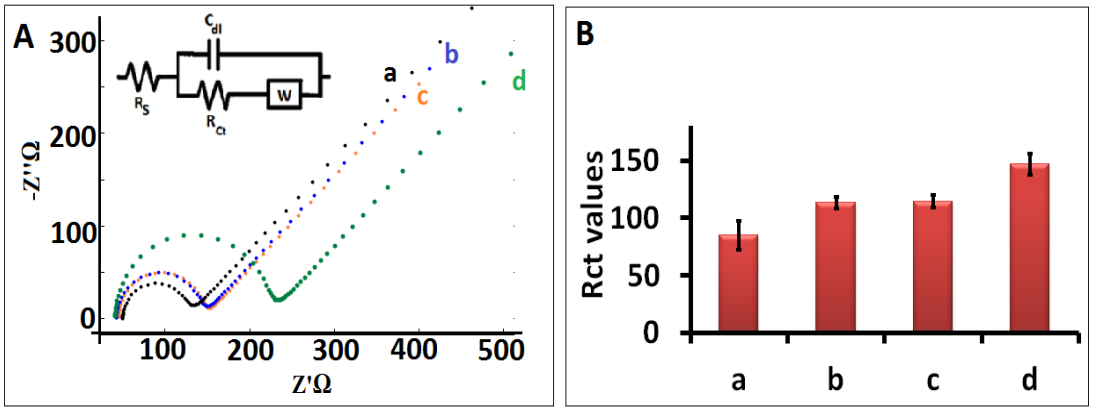

Figure 24 : Results of the Hybridization study of cell lysates via Nyquist plot diameters and column graphics . A section ; PGE coated with PPy (PGE/PPy,a), entrapped antimiR21 onto electrode surface with PPy matrix (PGE/PPy-antimiR, b), after hybridization (PGE/PPy-antimiR21/MCF-7, c) , non complemantary hybrid (PGE/PPy-antimir21/HUH-7,d) and B section respresents each sample Rct values with error bars

7.3 Calculation of Detection Limit

LOD is the lowest analyte to be detected statically. LOD can be calculated in different ways, with or without any instrument. When an instrument is used, signal/noise ratio is used and generally it is expected as 2:1 or 3:1.

Using excel LOD can be calculated easily. LOD of the study is calculated based on approchas following references [26, 125, 126] and found 1.4 pmol (Figure 25).

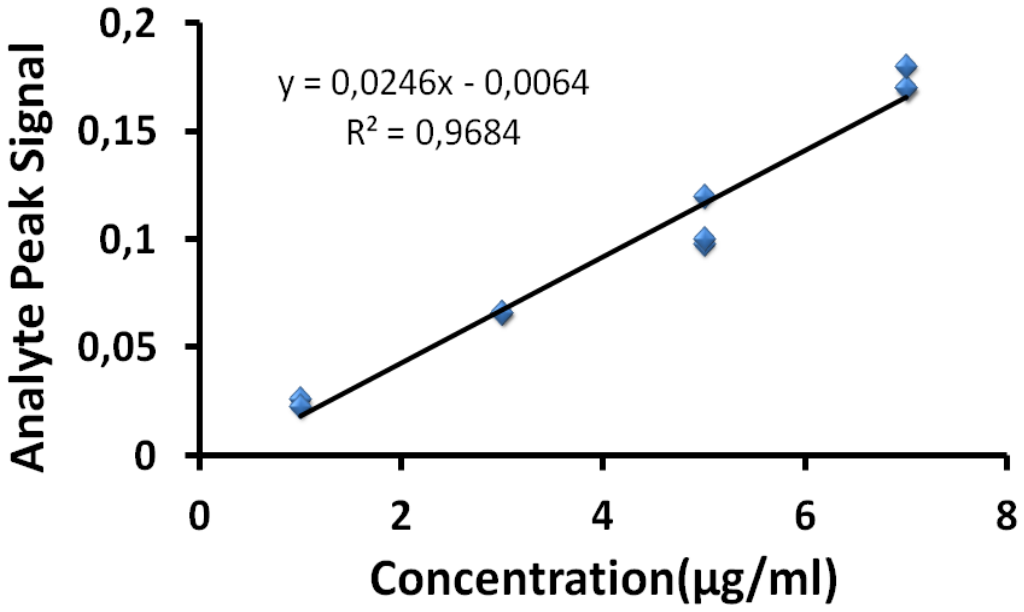

Figure 25 : Analyte response vs. concentration graphic

In calibration graphic the space between analyte concentration and sensor response gives information about LOD. The lowest concentration the sensor response is defined as the LOD. In calibration graphic, Substrat concentration vs sensor response relationship meeds to be lineer. Generally LOD is supposed to be lower than 10 µM. While calculating LOD through EIS Rct values are preferred to be low because when the R_{ct} value is low, the sensivity is high and signal to noise ratio is low. In enzyme sensor pH is a imporpant factor that need to be considered while calculating the LOD. Because pH effect the enzyme structure . pH effect the sensor response and cause of the biosensorsor failure

8. CONCLUSIONS

In addition to their possible use in anticancer theraphy, miRNAs are very good candidates for early cancer detection. Although there is an intensive research on miRNA detection, a standardized, simple, high-throughput and sensitive method has not been developed yet.

This study showed that MDB and EIS can be used as a tool in order to determine miR21 in MCF-7 cell lysates. PGE used as a disposable electrode . The biosensor was designed requires little time, no prufication, no complication. It can also be usen in real samples very effectively and easily. By this work, it was

presented for the first time a novel, ease-of-use, rapid, reproducible and selective PPy based miR21 biosensor with the detection limit of 1.4 pmol. Designed biosensor has superior advantages among other electrochemical assays such as being simple, reproducible, applicable to real samples without any need for pre-concentration or purification and having lower assay time. Morever, this assay could be applied for different miRNAs other than miR21 and integrated for other label-based electrochemical approaches as well as label-free ones.

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