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# Electrochemical based detection of microRNA, mir21 in breast cancer cells

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ABSTRACT

In this work, a novel electrochemical microRNA (miRNA) detection method based on enzyme amplified biosensing of mir21 from cell lysate of total RNA was demonstrated. The proposed enzymatic detection method was detailed and compared with the conventional guanine oxidation based assay in terms of detection limit and specificity. For the detection of mir21, capture probes and/or cell lysates were covalently attached onto the pencil graphite electrode (PGE) by coupling agents of *N*-(dimethylamino)propyl-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (NHS). Having immobilized the capture probe onto the surface of PGE, hybridization was achieved with a biotinylated (from its 3′ end) complementary target. Extravidin labeled alkaline phosphatase (Ex-Ap) binds to the biotinylated target due to the interaction between biotin–avidin and the enzyme converts electro-inactive alpha naphtyl phosphate (the substrate) to electro-active alpha naphtol (α-NAP, the product). α-NAP was oxidized at +0.23 V vs Ag/AgCl and this signal was measured by Differential Pulse Voltammetry (DPV). The signals obtained from α-NAP oxidation were compared for the probe and hybrid DNA. The specificity of the designed biosensor was proved by using non-complementary sequences instead of complementary sequences and the detection limit of the assay was calculated to be 6pmol for cell lysates.



## 1. Introduction

The new era in human genetics, miRNAs, are in the subgroup of naturally existing, small, non-coding ribonucleic acids (RNA) that regulate gene expression post-transcriptionally by binding with protein coding mRNAs (Shen et al., 2010; Zhu et al., 2008). The transcription of miRNAs (pri-miRNAs) is performed by RNA polymerase II or III in the nucleus. RNAse III endonuclease, Drosha, excises the pri-miRNA and processes an approximately 70 nucleotide length miRNA hairpin structure called pre-miRNA which is subsequently transported from nucleus to the cytoplasm via exportin 5 protein. In the cytoplasm, maturation of pre-miRNAs is provided by another RNase II endonuclease, Dicer. The mature miRNA is in the duplex form of 19–25 nucleotides in length (Kosaka et al., 2010; Sassen et al., 2008; Shen et al., 2010). The two strands of the miRNA duplex are called leading strand and passenger. The former one is bound by the ribonucleoprotein RNA-induced silencing complex (RISC) due to its weakly base paired 5′ end while the latter is degraded. The RISC complex integrated with miRNA hybridizes with target mRNA in order to regulate gene expression. The complementarity of the base pairs between the miRNA and mRNA is imperfect except in the region called "seed" at the 5′ end of the miRNA. Due to this imperfect-base pairing, not only can a single miRNA regulate more than one gene but also a gene can be targeted by more than one specific miRNA (Bandiera et al., 2010).

Microribonucleic acids have functions in several important biological events such as cell proliferation, apoptosis, differentiation, development and haematopoiesis metabolism (Bandiera et al., 2010; Cheng et al., 2005; Ferracin et al., 2010). It is still ambiguous whether the change in the miRNA expression level is the cause or consequence of occurrence of cancer (Sassen et al., 2008). Over the past decades, several groups have worked on miRNA profiling on different types of cancer. According to the findings, some types of miRNAs were seen to have a similar deregulation pattern across all types of cancers. For instance, in hepatocellular carcinoma mir26, mir26a, mir101 and mir122 are downregulated while mir200a, mir141 mir200c and mir200b are reported to be upregulated in ovarian carcinoma (Fornari et al., 2009; Iorio et al., 2007; Kota et al., 2009; Tsang et al., 2008).

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Due to the contribution of miRNAs to occurrence of diseases, their expression profiling is of great importance.

There are different strategies used for miRNA detection (Pritchard et al., 2012). The detection of hybridization is the underlying principle for most of the methods available for miRNA analysis (Castoldi et al., 2006; Cissell and Deo, 2009; Cissell et al., 2008; Fang et al., 2006; Li and Ruan, 2009; Persat and Santiago, 2011). These hybridization techniques can be subdivided into two groups as solid and solution phase hybridization methods. In the solid phase hybridization technique, a capture probe is immobilized onto a solid surface to be hybridized with its complementary target (Cissell and Deo, 2009). In the solution phase, probe and complementary targets are mixed and hybridized with each other and then the hybrid is immobilized to the electrode surface.

Recently studied solid phase methods on miRNA are Northern blotting, microarray, bioluminescent detection, surface enhanced Raman spectroscopy, surface plasmon resonance and electrochemical techniques. In the Northern blotting method, which is the golden standard for miRNA profiling, radiolabeled probes are hybridized with miRNA targets on a nitrocellulose membrane (Varallyay et al., 2008). Despite being the most standardized method, it is very time-consuming and not so efficient. In this regard, microarrays are on the rise since these systems are high throughput methods. Liang and co-workers designed a miRNA profiling microarray which works on the principle of hybridization of biotin labeled miRNAs with their complementary targets (Liang et al., 2005). In this study, the fluorescence signal of streptavidin labeled quantum dots that were bound to miRNAs due to the streptavidin–biotin interaction were detected by colorimetric methods.

In another microarray study, Locked Nucleic Acid (LNA) probes were used to increase melting point temperature of probe–miRNA heteroduplexes in order to increase sensitivity and stability (Castoldi et al., 2006; Li and Ruan, 2009). Driskell et al. developed a surface enhanced Raman spectroscopy platform for detection and classification of miRNA using silver nanorod substrates and partial least square discrimination analysis (Driskell et al., 2008). By polyadenylation and nanoparticle amplified surface plasmon resonance imaging, miRNAs were detected in femtomolar concentration range (Fang et al., 2006; Homola et al., 2010). Electrochemical detection is another solid phase miRNA analysis method and there are a few studies conducted regarding it. Laschi et al. investigated the properties of LNA, Peptide Nucleic Acids (PNA) and mir16 by using an enzyme amplified electrochemical detection procedure. The group used streptavidin coated magnetic beads for biotin tagged probe immobilization and a streptavidin conjugated enzyme for production of electrochemically active hydroquinone (Laschi et al., 2009). In another electrochemical approach, the aim was to detect mir122a based on guanine oxidation signal of the hybrid formed between the inosine substituted capture probe and mir122a target (Lusi et al., 2009).

The objective of this study is to design a rapid, selective and sensitive enzyme based electrochemical biosensor that is capable of detecting mir21 within real total RNA samples without any need for isolation or pre-concentration of mir21. In this regard, the oxidation signal of enzymatic reaction product, alpha naphtol (α-NAP), which is expected to be produced in the presence of hybrid, was detected by Differential Pulse Voltammetry (DPV) on a disposable PGE. Here, in this work, for the first time an enzyme based biosensor was designed for the detection of miRNA from cell lysates without any modification of sample. As a positive control, total RNA isolated from a breast cancer cell line that contains upregulated mir21 was used. The specificity of the assay was proved by non-complementary studies using mir21 free total RNA samples. The proposed enzyme based assay seems to provide more reproducible results for the detection of miRNA than conventional guanine oxidation based method for cell lysates. Additionally, the method can unambiguously distinguish between mir21 including samples and mir21 free samples while detection is impossible with the guanine based method due to very low signal (nanoampere scale).

## 2. Material and methods

### 2.1. Materials

The experiments were performed with a μ-Autolab type II digital potentiaostat/galvanostat using the GPES 4.8 software package (Eco Chemie, The Netherlands). Savitzy and Golay filter (Level 2) and moving average baseline correction with a peak width of 0.01 was used for treatment of raw data.

The three electrode system: pencil graphite electrode (PGE) as the working electrode (WE), Ag/AgCl electrode as the reference electrode (RE) and a platinum wire serving as the auxiliary electrode (AE) was used in connection with the Autolab software. Tombo HB model, 0.5 mm graphite lead was used as PGE. Methodological details about preparation of the WE are indicated in previously published papers (Del Giallo et al., 2005).

### 2.2. Oligonucleotides and chemicals

The HPLC purified 22-mer synthetic oligonucleotides of mir21 were purchased from Alpha DNA (Canada) in lyophilized form and have the following sequences;

mir 21 (RNA):
5′-UAG CUU AUC AGA CUG AUG UUG A-3′
mir 21 (DNA):
5′-TAG CTT ATC AGA CTG ATG TTG A-3′
Antimir 21 (Inosine substituted):
5′-TCA ACA TCA ITC TIA TAA ICT A-3′
Antimir 21 (biotin and inosine conjugated):
5′-TCA ACA TCA ITC TIA TAA ICT A–biotin-3′
Non-Complementary—mir 192:
5′-CUG ACC UAU GAA UUG ACA GCC-3′

Chemicals required for preparation of 0.5 M acetate buffer solution containing 20 mM NaCl (ABS, pH 4.8), 0.05 M phosphate buffer solution (PBS, pH 7.4) and diethanolamine (DEA) buffer (pH 9.6) were bought from Sigma Aldrich. NHS and EDC were also purchased from Sigma Aldrich. Alkaline phosphatase (Ex-Ap) was from Pierce. All chemicals used in this study were of analytical grade and the experiments were conducted at room temperature. Synthetic oligonucleotides were prepared in PBS and for further dilutions PBS containing either 20 mM or 500 mM NaCl was used.

Throughout this study, the detection of mir21 was done not only by synthetic oligonucleotides but also by cell lysates. For this reason, three different total RNA samples from two different cell lines (MCF-7 and 293-HEK) were provided by various labs in the Pharmacy Department of Ege University, Izmir. For all cell lines, cultivation was done in a humidified incubator at 37 °C with 5% $CO_2$. Cells were cultured in DMEM supplemented with L-glutamine (2 mmol/L), 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum. Tissue culture reagents were purchased from Biological Industries. The cell lysate including upregulated levels of mir21 is from MCF7 human breast adenocarcinoma cell line and the first non-complementary sample (NC-1) that is from 293-HEK human embryonic kidney cell line was extracted with the Trizol reagent (Invitrogen).

Briefly, cells were lysed with Trizol and after 5 min of incubation, 0.2 mL chloroform per mL of Trizol was added into the samples. Centrifugation was carried out at 12,000 *g* for 15 min at

4 °C and following vigorous shaking of samples, total RNA was obtained in the upper aqueous phase. Total RNA was precipitated by addition of isopropyl alcohol, centrifuged and washed once with ethanol. Finally the pellet was air-dried and dissolved in RNase-free water. Non-complementary sample-2 (NC-2) was again obtained from 293-HEK cell lines but this time with a different extraction method and prepared by different researchers. In this extraction, a specific RNA isolation kit was used according to the manufacturer's instructions (EZNA).

### 2.3. Procedure

Throughout this study, several methodologies were developed. However, two of them, guanine and alpha naphtol oxidation based methods were detailed.

#### 2.3.1. Procedure based on guanine signal

First step of the experiments based on guanine signal was pretreatment of PGEs by applying +1.4 V for 30 s in ABS. Next, probe solution having desired concentration was prepared in PBS and immobilization of probe onto PGE surface was achieved by passive adsorption for 20 min. In order to prevent non-specific binding, washing step was performed in PBS. Half of the probe attached PGEs were dipped into the target solution, while others were exposed to buffer solution for 20 min to achieve hybridization/pseudo-hybridization. After hybridization, another washing step was performed in PBS. The oxidation signals of guanine bases were measured by DPV at 50 mV amplitude scanning from +0.75 to +1.4 V in ACB.

#### 2.3.2. Procedure based on alpha naphtol signal

Both synthetic and cell lysates were examined by the procedure based on α-NAP oxidation signal and the steps of the method are shown in Scheme 1. Basically the procedure was composed of seven steps: electrochemical and covalent activation of the PGE surface, probe immobilization onto the surface of PGE electrode, hybridization, biotin–streptavidin interaction, enzyme–substrate interaction and the measurement of the product signal.

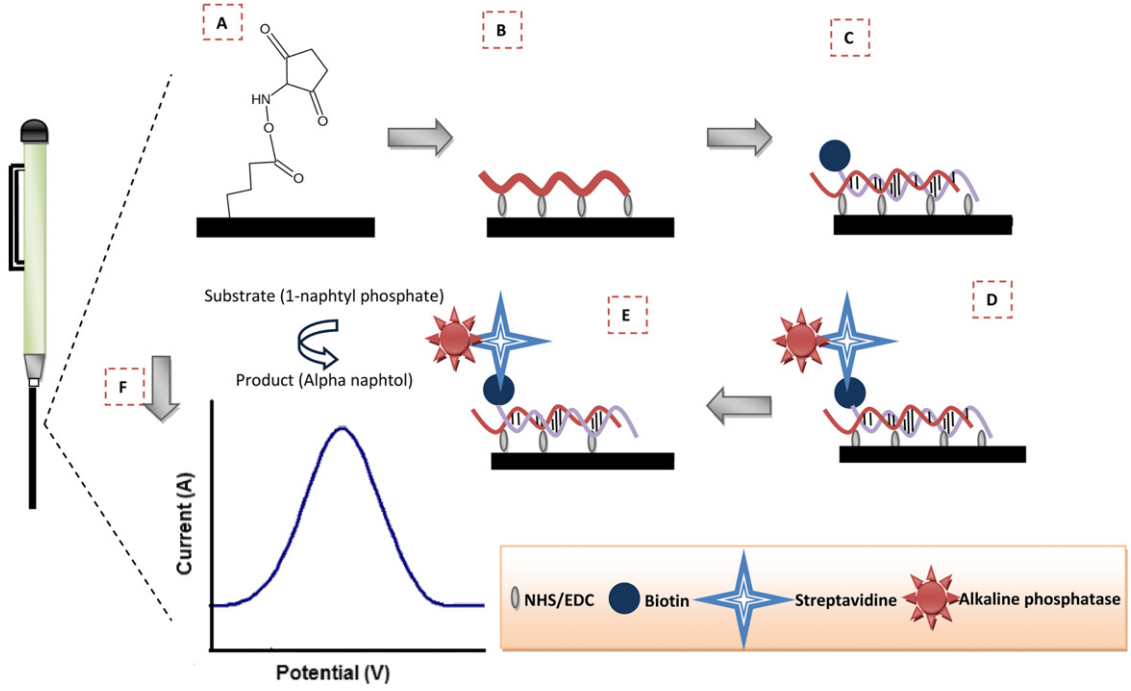
*2.3.2.1. Covalent activation of the PGE surface.* Our results indicated that attachment of total RNA onto the PGE surface was possible neither by adsorption nor application of positive potential to the electrode (data not shown). The only way for immobilization of total RNA onto the electrode surface was by covalent attachment. First, the surface was electroactivated via application of +1.4 V for 30 s versus Ag/AgCl in ABS. Electroactivation or pre-treatment introduces the carboxylic acid groups to the surface and in the presence of EDC and NHS, these groups are converted to succinimidyl ester (–COOSuc) (Sam et al., 2010). Hence, the number of carboxylic acid groups is of great importance for improving the performance of the sensor.

The electroactivation of PGE was done at different potentials varying from +0.5 V to+1.6 V. Above +1.4 V, the surface of PGE was destroyed and overload recorded. The highest oxidation charge value was obtained at 1.4 V (see supplementary material).

Next, prior to probe immobilization, electroactivated PGEs were dipped into a solution containing 8 mM NHS and 5 mM EDC and kept there for 1 h to achieve covalent surface activation. Then PGEs were washed in PBS once.

*2.3.2.2. Probe immobilization onto the surface of electrode.* Having modified the PGE surface, the probe (synthetic (mir21)/cell lysate), became capable of attaching to the surface via amide linkage between the amine groups in the guanine bases of probe (N7 position of guanine) and –COOSuc (Pividori et al., 2000). According to the specified concentration, probe stock solution was diluted with PBS and the PGEs were dipped into the PCR tubes containing 30 μL probe solution and were kept there for 20 min.

*2.3.2.3. Hybridization.* After immobilization of probe (synthetic mir21/mir21 including cell lysate) onto the PGE surface, half of




**Scheme 1.** Sketch of steps involved in (A) surface activation of PGE with NHS/EDC, (B) probe immobilization on to the surface of PGE, (C) hybridization of probe with biotinylated target, (D) Ex-Ap–biotin interaction, (E) enzyme substrate interaction and production of α-NAP and (F) voltammetric measurement of oxidation signal of α-NAP.

the probe covered PGEs were dipped into biotin labeled antimir21 target solution and others into blank hybridization buffer and all electrodes were kept in those solutions for 20 min to achieve hybridization/pseudo-hybridization. Although the probe was lying flat on PGE, it can hybridize with its complementary target since the groups of guanine that bind to the PGE surface and the groups that make H bonds with cytosine are different. Hybridization occurs between C-2 carbonyl, N-3 amine and C-6 groups of guanine and cytosine, while the interaction between guanine and –COOSuc is from the N7 position of guanine (Pividori et al., 2000) (supplementary information, Fig. S1). The hybridization was monitored by comparing the signals of hybridized and pseudo-hybridized electrodes.

*2.3.2.4. Enzyme interaction.* After hybridization of mir21 with biotin labeled antimir21, the duplex interacted with Ex-Ap. The enzyme solution was diluted by DEA solution (pH 9.6) including BSA (10 mg/mL) so as to have a final concentration of 1 μL enzyme in 1 ml DEA and then interacted with biotin for 20 min. In order to prevent non-specific binding, electrodes were washed for 30 s by mixing. Afterwards, until measurements were carried out, PGEs were kept in DEA solution to prevent the transducer surface from getting dried out.

*2.3.2.5. Enzyme substrate interaction and measurement of the product signal.* Enzyme modified PGE surfaces were exposed to 1-naphtyl phosphate solution (1 mg/mL substrate solution in DEA buffer) at open circuit potential by mixing for 3 min. The electrochemical oxidation signal of the reaction product of alkaline phosphatase, α-NAP, was detected by DPV at +0.23 V versus Ag/AgCl electrode after the interaction of substrate with the enzyme. The measurements were done inside the cell containing substrate solution.

## 3. Results and discussion

Detection of mir21 from cell lysate requires a very sensitive detection method since total RNA includes a small amount of mir21 as well as other types of miRNAs. In order to carry out the objective of this study, detecting mir21 from cell lysate by electrochemical biosensors without any need for modification such as pre-concentration, different experimental approaches were tested. An enzyme based method was proposed since the guanine based method produced irreproducible nanoampere scale oxidation signals for cell lysate experiments. The obtained results from these two methods were categorized into two according to the types of miRNAs used (synthetic or cell lysate) and the types of signals recorded.

### 3.1. Experiments conducted with synthetic miRNAs

By the experiments conducted with synthetic oligonucleotides, both guanine oxidation signals and α-NAP oxidation signals were reproducible. So, these experiments were detailed in two categories according to the electrochemical signal recorded.

#### 3.1.1. Experimental results based on guanine oxidation signal

The first experiments of guanine oxidation based method were conducted by synthetic oligonucleotides. Having seen the positive results with synthetic oligonucleotides, cell lysates were examined in terms of their mir21 content. Owing to the fact that inosine is a non-electroactive substitute of guanine and is used as the indicator of hybridization due to the increase in guanine oxidation signal after hybridization (Ozkan-Ariksoysal et al., 2008; Wang et al., 1998), in this work, synthetic inosine substituted antimir21 was designed although the complementary targets mir21 DNA (Target 1—$T_1$) and mir21 RNA (Target 2—$T_2$) contained guanines instead of inosines.

As seen in Fig. 1 the guanine oxidation signal of inosine substituted antimir21 (Probe-P) was not significant in comparing $T_1$ and $T_2$ since the inosine substituted probe could not produce oxidation signals. Similarly, the guanine oxidation signals of hybrids antimir21–mir21 DNA (hybrid 1—$H_1$) and antimir21–mir21 RNA (hybrid 2—$H_2$) were also higher than the signals of the probe.

After hybridization, the guanine oxidation signal increased due to the guanines existing in target sequences. On the other hand, if the oxidation signal of targets and hybrids were compared, it was seen that, due to hydrogen bond formation between guanines of the target and cytosines of the probe during hybridization, guanines became inaccessible for the oxidation resulting in a decrease in guanine oxidation signal. Hybridization was also proved by the decrease in guanine signals after hybridization of mir21 DNA/ mir21 RNA with inosine substituted antimir21. Generally, in the hybridization experiments, probe is immobilized to the surface and then target is introduced to the probe covered electrodes. In one set of our experiment, we immobilized the target to the surface first and then we sent the probes. Here, the probes were mir21 DNA and mir21 RNA and the target was inosine substituted antimir21.

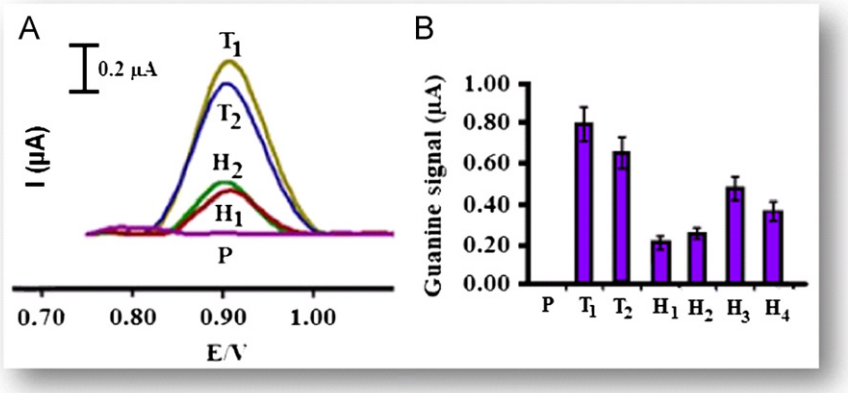



**Fig. 1.** Differential pulse voltammograms (A) and histograms with error bars (B) with synthetic oligonucleotides on PGEs with antimir21 probe (*P*), mir21 DNA (*T*1), mir21 RNA (*T*2), hybrid of *P* and *T*1 (H1), hybrid of *P* and *T*2 (*H*2), hybrid of *T*1 and *P* (reverse order as in *H*1 probe was used as target, target was used as probe, *H*3) and hybrid of *T*2 and *P* (reverse order as in *H*2 probe was used as target, target was used as probe, *H*4). Experimental procedure was detailed in Section 2.3.1.

Corresponding hybrids are mir21 DNA–antimir21 (hybrid 3—$H_3$) and mir21 RNA—antimir21 (hybrid 4—$H_4$) as shown in Fig. 1.

Having seen hybridization with synthetic oligonucleotides, we determined the detection limit of the assay. For this purpose, mir21 RNA concentration was changed while antimir21 probe concentration was kept constant at 5 μg/mL. The minimum detectable concentration for mir21 RNA, was found to be 0.67 μg/mL (equivalent to 1 μM according to the molecular weight data of mir21 represented in the data sheet provided by Alpha DNA), and the detection limit was calculated to be 4 pmol (the used volume and concentration for detection was 40 μL and 1 μM respectively). As seen in Fig. 2, after 0.67 μg/mL, no significant rise in the guanine oxidation signal was detected. Lusi et al. recorded a 0.02 picomole detection limit for their assay in which inosine substituted probe was used (Lusi et al., 2009). For their case the guanine oxidation signal for inosine substituted probe was found to be 60 nA, whereas in our procedure, it was almost zero.

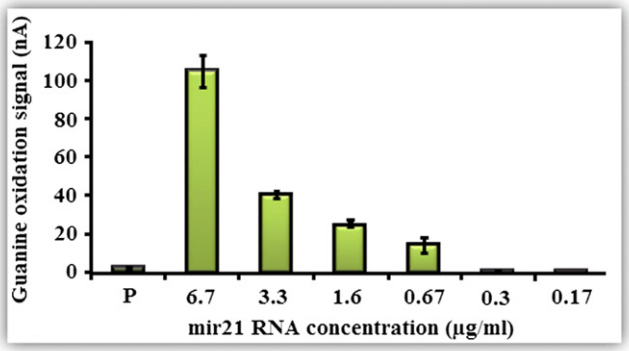



**Fig. 2.** Histograms for the concentration study of mir21 RNA representing guanine oxidation signals obtained in PBS for 5 μg/mL inosine substituted antimir21 probe ($P$) and mir21 RNA target at various concentrations between 6.7 μg/mL and 0.17 μg/mL.

Although this guanine based method enabled a very low detection limit, this procedure did not work for cell lysates. The guanine signals recorded before and after hybridization of total RNA and the reproducibility were so low that the method was concluded to be inefficient for unambiguously distinguishing between probe and hybrid for cell lysates. So, for the direct detection of mir21 from cell lysate, enzymatic detection procedure was implemented.

### 3.1.2. *Experimental results based on α-NAP oxidation signal*

Enzymes as signal amplifiers have been used for several genosensor assays for decades (Carpini et al., 2004; Del Giallo et al., 2005). In this work, Ex-Ap was used to detect the α-NAP that is the oxidation product of 1-naphtyl phosphate. Since streptavidin has a high affinity to biotin, the enzyme was expected to govern the oxidation reaction in the case of biotin substituted target addition; in other words, when the hybridization occurs. For this reason, the product signal recorded for the hybrid must be much higher than the one recorded for the probe.

As shown in Fig. 3, for both mir21 DNA ($P_1$) and mir21 RNA ($P_2$), the oxidation signal of α-NAP was higher in duplex form ($H_1$ and $H_2$) than in the probe itself due to biotin conjugate in antimir21. Theoretically, there should be no signal concerning the probe due to lack of biotin and thereby Ex-Ap product of alpha naphtyl phosphate. But it was seen that the product signal for probe was nearly one-half of the α-NAP signal of the hybrid.

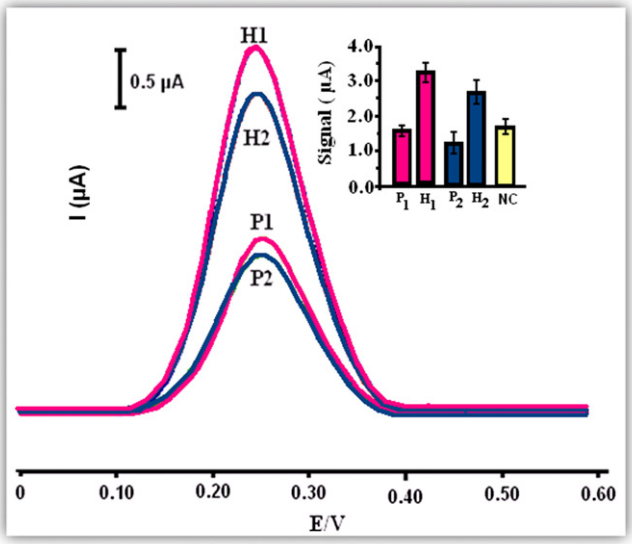



**Fig. 3.** α-NAP oxidation signals obtained in α-naphtyl phosphate solution by DPV following the experimental procedure detailed in 2.3.2 belonging to mir21 DNA ($P_1$), mir21 RNA ($P_2$), hybrid of $P_1$ and biotin conjugated antimir21 ($H_1$), hybrid of $P_2$ and biotin conjugated antimir21 ($H_2$), mir 192 and biotin conjugated antimir21 (non-complementary-NC).

This unexpected α-NAP signal recorded for the probe may be explained by non-specific attachment of Ex-Ap to the surface of PGEs due to the fact that probe did not fully cover the PGE surface. This speculation was checked by experiments in which Ex-Ap was attached to the NHS/EDC covered blank (without real or synthetic oligonucleotides on them) PGEs and it was seen that the enzyme itself also binds to the surface of the electrodes. From this observation, bovine serum albumin (BSA) and ethanolamine were used as blocking agents for filling empty spaces remaining from the probe. However, the difference in the amplitudes of α-NAP signals obtained for probe and hybrid could not be increased and even the hybridization diminished (data not shown) so the experiments were conducted without blocking agents.

Any increase in mir21 concentration decreased the blank α-NAP oxidation signal (signal obtained for probe) up to some extent (data not shown) but since the aim was lowering the detection limit as much as possible, the probe concentration was chosen to be the minimum concentration, 5 μg/mL that will enable us to distinguish the product signal between the probe and hybrid form. At this concentration, target concentration was used as 0.67 μg/mL as specified to be the detectable minimum concentration in guanine based experiments. Non-complementary sequence, mir192 was used to prove the specificity of the assay. When it was hybridized with biotinylated antimir21, the signal remained the same as proof that no hybridization occurred between the two oligonucleotides and that the designed biosensor was specific to mir21 only.

### 3.2. *Experiments conducted with total RNA extracted from cell lysates*

Having obtained reproducible results with synthetic oligonucleotides, enzyme based experiments were conducted with total RNA extracted from cell lysates. The first experimental set was constructed to specify the minimum detectable mir21 concentration in total RNA. To realize this, the total RNA concentration was changed from 0.1 to 3 μg/mL when concentration of

antimir21 biotin was kept constant at the previously specified concentration of 0.67 μg/mL.

As is presented in Fig. 4, the maximum α-NAP signal difference between probe (B) and hybrid (A) was achieved at the concentration value of 1 μg/mL. In other words, the detection limit for the enzyme based designed biosensor is less than 6 pmol (According to the data sheet obtained from Alpha DNA (Canada), the molecular weight of mir21 is 6652 g/mol, the volume required for detection is 40 μL and the total RNA contains other miRNA types as well as mir21) since total RNA does not fully consist of mir21.

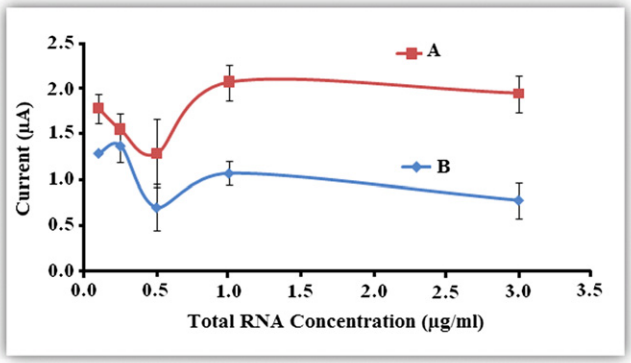



**Fig. 4.** α-NAP oxidation signals obtained in α-naphtyl phosphate solution by DPV following the experimental procedure detailed in 2.3.2 belonging to concentration study of total RNA at constant biotinylated antimir21 concentration (0.67 μg/mL) before hybridization (B) and after hybridization with biotinylated antimir21 (A).

As is known, some total RNA samples may contain an excessive amount of mir21 due to the isolated tissue. For instance, in breast cancer, mir21 is known to be upregulated and the concentration is expected to be higher in an extract obtained from a person suffering from breast cancer than the concentration of mir21 in the extract obtained from a healthy person (Yang et al., 2009). For our work, total RNA extracted from MCF-7 cancer cell line was used as the positive sample since it contains upregulated levels of mir21 due to the characteristics of breast cancer. The total RNA samples extracted from 293-HEK cell line by two different procedures and in two different labs were the negative controls (non-complementary 1-NC-1 and non-complementary 2-NC-2) due to its mir21 free nature.

The specificity of the assay is important as much as the detection limit. In biosensor applications, it is desired to have target specific detection. In our case, the designed biosensor must be mir21 specific and must not produce any signals due to hybridization occurring between biotinylated Antimir21 and other microRNAs included in cell lysates. This was checked by two non-complementary mir21 free cell lysates (NC-1 and NC-2).

According to the results presented by Fig. 5, for NC-1 and NC-2, α-NAP oxidation signals for probe (Fig. 5A) and hybrid (Fig. 5B) were nearly the same which means no hybridization occurred between antimir21 and other miRNAs (different from mir21) present in the cell lysate. This shows how much the assay is specific to the concerned microRNA. Although NC-1 and NC-2 total RNA were extracted from the same cell line (293-HEK human embryonic kidney cell line) by two different labs and following two different procedures, the signals obtained for both samples at the same conditions showed similar pattern indicating that the results are reproducible with the average RSD value of 5% in terms of both time and different researchers.

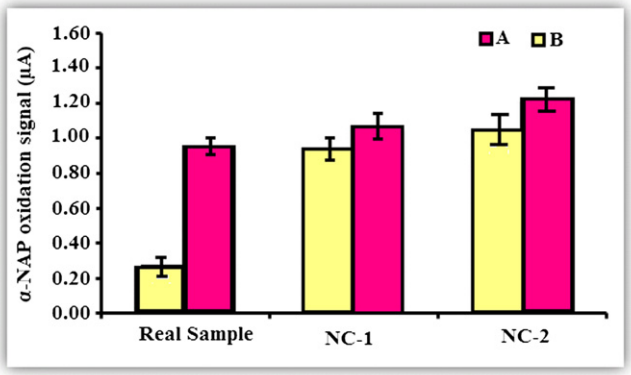



**Fig. 5.** α-NAP oxidation signals of cell lysate and mir21-free non-complementary samples obtained in α-naphtyl phosphate solution by DPV following the experimental procedure detailed in 2.3.2 before (A) and after hybridization with biotin conjugated antimir21 (B).

## 4. Conclusions

The initiation and progression of malignant behaviors of cancer were associated with microRNAs by several comprehensive studies (Ferracin et al., 2010; Molnar et al., 2008; Sassen et al., 2008; Wu et al., 2010). It is proved by extensive microRNA expression profiling methods that some cancer samples and their accompanying healthy tissues have specific signatures for each cancer type. Under the circumstances, detection of microRNAs for prognosis of cancer comes into prominence.

This work aimed to design for the first time an enzyme based biosensor that is capable of detecting miR21, a type of microRNA that is known to be upregulated in breast carcinoma tissues (Yang et al., 2009), from cell lysate of total RNA. By its detection limit of 6 pmol, the designed biosensor represents a novel alternative to other techniques used for microRNA screening. As obtained experimental results indicate, by the use of enzyme amplification, the detection of mir21 from total RNA isolated from breast cancer cell lines become possible due to reproducible signal values.

Specificity of the developed biosensor was controlled by utilizing non-complementary RNA from a mir21 free cell line and the detection of alpha naphtol signals for probe and hybrid indicated that hybridization did not occur. The constructed biosensor assay has several advantages such as being accurate, reproducible, robust and not so time-consuming when compared to northern blotting.

Also, it is applicable to other real samples of total RNA that are extracted from various cancer tissues or cell lines and can be used for detection of other types of microRNAs.

So far, different methods have been developed for miRNA analysis but still new methods are needed that are reliable, standardized and also capable of establishing multiplex detection of various types of miRNAs at the same time. Being user friendly, inexpensive and with other advantages, biosensors for miRNA detection will be indispensible tools among other profiling methods for medical diagnosis in the future.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.05.031.

## References


Bandiera, S., Hatem, E., Lyonnet, S., Henrion-Caude, A., 2010. Clinical Genetics 77 (4), 306–313.

Carpini, G., Marrazza, G., Mascini, M., 2004. Proceedings, 82–87.

Castoldi, M., Schmidt, S., Benes, V., Noerholm, M., Kulozik, A.E., Hentze, M.W., Muckenthaler, M.U., 2006. RNA—A Publication of the RNA Society 12 (5), 913–920.

Cheng, A.M., Byrom, M.W., Shelton, J., Ford, L.P., 2005. Nucleic Acids Research 33 (4), 1290–1297.

Cissell, K.A., Deo, S.K., 2009. Analytical and Bioanalytical Chemistry 394 (4), 1109–1116.

Cissell, K.A., Rahimi, Y., Shrestha, S., Hunt, E.A., Deo, S.K., 2008. Analytical Chemistry 80 (7), 2319–2325.

Del Giallo, M.L., Ariksoysal, D., Marrazza, G., Mascini, M., Ozsoz, M., 2005. Analytical Letters 38 (15), 2509–2523.

Driskell, J.D., Seto, A.G., Jones, L.P., Jokela, S., Dluhy, R.A., Zhao, Y.P., Tripp, R.A., 2008. Biosensors and Bioelectronics 24 (4), 917–922.

Fang, S.P., Lee, H.J., Wark, A.W., Corn, R.M., 2006. Journal of the American Chemical Society 128 (43), 14044–14046.

Ferracin, M., Veronese, A., Negrini, M., 2010. Expert Review of Molecular Diagnostics 10 (3), 297–308.

Fornari, F., Gramantieri, L., Giovannini, C., Veronese, A., Ferracin, M., Sabbioni, S., Calin, G.A., Grazi, G.L., Croce, C.M., Tavolari, S., Chieco, P., Negrini, M., Bolondi, L., 2009. Cancer Research 69 (14), 5761–5767.

Homola, J., Sipova, H., Zhang, S.L., Dudley, A.M., Galas, D., Wang, K., 2010. Analytical Chemistry 82 (24), 10110–10115.

Iorio, M.V., Visone, R., Di Leva, G., Donati, V., Petrocca, F., Casalini, P., Taccioli, C., Volinia, S., Liu, C.G., Alder, H., Calin, G.A., Menard, S., Croce, C.M., 2007. Cancer Research 67 (18), 8699–8707.

Kosaka, N., Iguchi, H., Ochiya, T., 2010. Cancer Science 101 (10), 2087–2092.

Kota, J., Chivukula, R.R., O'Donnell, K.A., Wentzel, E.A., Montgomery, C.L., Hwang, H.W., Chang, T.C., Vivekanandan, P., Torbenson, M., Clark, K.R., Mendell, J.R., Mendell, J.T., 2009. Cell 137 (6), 1005–1017.

Laschi, S., Palchetti, I., Marrazza, G., Mascini, M., 2009. Bioelectrochemistry 76 (1–2), 214–220.

Li, W., Ruan, K.C., 2009. Analytical and Bioanalytical Chemistry 394 (4), 1117–1124.

Liang, R.Q., Li, W., Li, Y., Tan, C.Y., Li, J.X., Jin, Y.X., Ruan, K.C., 2005. Nucleic Acids Research 33 (2).

Lusi, E.A., Passamano, M., Guarascio, P., Scarpa, A., Schiavo, L., 2009. Analytical Chemistry 81 (7), 2819–2822.

Molnar, V., Tamasi, V., Bakos, B., Wiener, Z., Falus, A., 2008. Seminars in Cancer Biology 18 (2), 111–122.

Ozkan-Ariksoysal, D., Tezcanli, B., Kosova, B., Ozsoz, M., 2008. Analytical Chemistry 80 (3), 588–596.

Persat, A., Santiago, J.G., 2011. Analytical Chemistry 83 (6), 2310–2316.

Pividori, M.I., Merkoci, A., Alegret, S., 2000. Biosensors and Bioelectronics 15 (5–6), 291–303.

Pritchard, C.C., Cheng, H.H., Tewari, M., 2012. Nature Review Genetics 13 (5), 358–369.

Sam, S., Touahir, L., Andresa, J.S., Allongue, P., Chazalviel, J.N., Gouget-Laemmel, A.C., de Villeneuve, C.H., Moraillon, A., Ozanam, F., Gabouze, N., Djebbar, S., 2010. Langmuir 26 (2), 809–814.

Sassen, S., Miska, E.A., Caldas, C., 2008. Virchows Archiv 452 (1), 1–10.

Shen, X.J., Huang, Y., Zou, Q.A., Zhao, Q.L., 2010. Russian Journal of Bioorganic Chemistry 36 (6), 684–689.

Tsang, T.Y., Tang, W.Y., Tsang, W.P., Co, N.N., Kong, S.K., Kwok, T.T., 2008. Apoptosis 13 (9), 1135–1147.

Varallyay, E., Burgyan, J., Havelda, Z., 2008. Nature Protocols 3 (2), 190–196.

Wang, J., Rivas, G., Fernandes, J.R., Paz, J.L.L., Jiang, M., Waymire, R., 1998. Analytica Chimica Acta 375 (3), 197–203.

Wu, Q.A., Li, H.L., Lu, J.F., Ge, Q.Y., Lu, Z.H., 2010. Chinese Science Bulletin 55 (31), 3517–3526.

Yang, Y., Chaerkady, R., Beer, M.A., Mendell, J.T., Pandey, A., 2009. Proteomics 9 (5), 1374–1384.

Zhu, S.M., Wu, H.L., Wu, F.T., Nie, D.T., Sheng, S.J., Mo, Y.Y., 2008. Cell Research 18 (3), 350–359.