Biosensors and Bioelectronics 48 (2013) 165–171

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A new insight into electrochemical microRNA detection: A molecular caliper, p19 protein

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ARTICLE INFO

Article history:
Received 1 February 2013
Received in revised form
28 March 2013
Accepted 8 April 2013
Available online 20 April 2013

Keywords:
p19 protein
mir21
Electrochemical DNA biosensor
Differential pulse voltammetry (DPV)
Breast cancer

ABSTRACT

microRNA (miRNA) has drawn a great attention in biomedical research due to its functions on biological processes. Detection of miRNAs is a big challenge since the amount present in real samples is very low and the length of them is short. In this study, for the first time an electrochemical biosensor for detection of mir21 using the oxidation signal of protein 19 (p19) as a molecular caliper was designed. The proposed method enables detection of mir21 in direct, rapid, sensitive, inexpensive and label-free way. Binding specificity of the p19 to 20–23 base pair length double stranded RNA (dsRNA) and direct/water-mediated intermolecular contacts between the fusion protein and miRNA allows detection of miRNA–antimiRNA hybrid structure. The detection of mir21 was achieved in picomole sensitivity through the changes of intrinsic p19 oxidation signals observed at +0.80 V with Differential Pulse Voltammetry (DPV) and the specifity of the designed sensor was proved by control studies.

1. Introduction

Owing to its astonishing nature, RNA silencing or inhibition of gene expression in a sequence specific manner lead to many studies conducted by investigators across various disciplines. Evolutionary conserved RNA silencing mechanism basically consists of three steps; scission of double-stranded RNA (dsRNA), formation of RNA induced silencing complex (RISC) and degradation of mRNA (Lesicka et al., 2004). In the first step, synthesized or introduced double stranded RNA is cleaved into 20–30 nucleotide long small dsRNAs by a ribonuclease III enzyme named as, Dicer. Next, these small duplexes are disentangled and one of the strand which is called the “leading” or the “guide” strand gets incorporated into RNA induced silencing complex (RISC). Finally, an endonuclease enzyme cleaves the target mRNA—in the case of perfect base pairing between the target mRNA and the leading strand. If there is an imperfect hybridization/complementarity between two, the protein expression is repressed (Siomi and Siomi, 2009).

Two types of small (21–23 base pair long) non-coding RNAs; microRNAs (miRNAs) and short interfering RNAs (siRNAs) plays an active role in RNA silencing mechanism. Biogenesis and mechanism of both types of RNAs depend on two protein families; an RNAse III family member Dicer enzymes and argonaute proteins. The structure and origin of these two small RNAs differ from each other. An evolutionary conserved, genome-encoded triggers, miRNAs are endogenous while siRNAs are exogenous and responsible for mediating immune response in case of foreign nucleic acid invasion such as viruses, transposons and transgenes (Carthew and Sontheimer, 2009). Additionally, siRNAs are supremely complementary to their RNA targets while miRNAs are imperfectly match to their targets due to often having mismatches, bulges and loops (Khan et al., 2011). The main difference between siRNAs and miRNAs is that miRNAs are generally single stranded while siRNAs could exist in both single- and double-stranded forms (Baulcombe and Molnar, 2004). Various biological systems such as hematopoiesis (Bissels et al., 2012), cell differentiation (Zhang et al., 2012), proliferation (Lukiw et al., 2010) and apoptosis (Glorian et al., 2011) are regulated by miRNAs. miRNAs have also been identified in normal and malignant cells. They could be oncogenic or suppressors of tumor formation through regulation of expression of target genes. Deregulation of miRNA expression is assumed to be an implication for some cancers (Sassen et al., 2008).

miRNAs could be either up or down regulated in types of cancers and figuring out their regulation pattern is of great importance since it could provide information about their potential roles in tumor initiation, progression of invasion and metastasis. miRNA has been reported to be highly expressed in various cancers such as breast (Hong et al., 2012; Tjensvoll et al., 2012) and lung (Jang et al., 2012) cancers, and down regulated in prostate cancer (Ru et al., 2012). Since deregulation of miRNA is involved in

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http://dx.doi.org/10.1016/j.bios.2013.04.011

pathogenesis of various disorders including human cancers, miRNAs are considered to be potential biomarkers for the diagnosis and treatment of cancer. Therefore, a sensitive detection method for identification and/or quantification of miRNA is of great importance. Developing such a method is still challenging due to short length and similarities of miRNAs. Up to date, various methods have been demonstrated for this purpose.

Among them, despite their low sensitivity and long assay time requirements, northern blotting is used as a golden standard (Qavi et al., 2010; Valoczi et al., 2004). Microarrays are also commonly used since they enable multiplex detection, relative discrimination of expression levels of miRNA between two samples, identification of precursors and mature forms.

However, they present stringency drawbacks (Li et al., 2010; Liu et al., 2008; Nelson et al., 2004). Quantitative real time polymerase chain reaction (qRT-PCR) is a technique applied for measuring expression levels of miRNA (Varkonyi-Gasic et al., 2007) due to low melting point temperatures resulted from shorter lengths of miRNAs than primers used in conventional PCR, there exist some challenges to overcome (Benes and Castoldi, 2010; Qavi et al., 2010). In order to overcome these limitations, alternative methods have been developed. These include nanotechnology based techniques such as electro catalytic nanoparticle based miRNA detection with amperometric measurement (Gao and Yang, 2006), the chemiluminescence technique (Bi et al., 2011), an array-based method for miRNA expression profiling using locked nucleic acid capture probes (Castoldi et al., 2008). Profiting from the advantages offered by nanotechnology, optical (Cissell et al., 2008; Sipova et al., 2010; Zhang and Zhang, 2012) and electrical (Dorvel et al., 2012; Fan et al., 2007; Francesca Bettazzi et al., 2013; Gu et al., 2012; Poehlmann and Sprinzl, 2010; Zhang et al., 2009; Zhou et al., 2012) biosensors with their high sensitivity, applicability to real samples without any amplification, short assay time, ease of use and adoptability to point-of-care testing, have been increasingly used as promising miRNA detection methods (Kilic et al., 2012).

The objective of this study is to provide a novel electrochemical biosensor approach for direct detection of mir21, a cancer biomarker to be up regulated in breast cancer cells (Zhu et al., 2008). Herein, a sensitive assay for mir21 detection was developed using protein 19 (p19). The analytical signal used during detection belongs to the homodimeric, Carnation Italian Ringsport Virus (CIRV) encoded, 19 kDa fusion protein named p19 which is a RNA silencing suppressor (Vargason et al., 2003). By pairing up the siRNA/miRNA recognition capability of p19 protein with electrochemistry, developed biosensor offers sensitive detection in relatively short assay time without using any labels. P19 behaves like a molecular caliper of dsRNA and sequesters miRNAs in a size dependent, sequence independent manner. Hybridization of a miRNA probe and its target creates dsRNA structure and this formation firmly binds p19 protein. Additionally, p19 protein does not interact with DNA due to lack of 2′-OH groups in the DNA structure. Possessing the bracketing function, the end capping tryptophan inside the p19 protein are reported to be moliminous in terms of RNA recognition and RNA silencing suppression (Baulcombe and Molnar, 2004; Ye et al., 2003). Based on this feature and previously characterized oxidation signal of tryptophan (Ozcan and Sahin, 2012), designed biosensor exemplifies for the first time the uses of electrochemistry in miRNA detection with the help of intrinsic characteristic of p19 protein and eliminates the drawbacks such as; being time consuming, need of radioactive labeling, pre-amplification of miRNA prior to detection, overnight hybridization and post-hybridization (See Table S1 for making comparison between different miRNA detection methods). The unique property of p19 protein fascinated another group who designed a three-mode electrochemical biosensor for miRNA detection upon p19 protein binding and its displacement (Mahmoud Labib et al., 2013). Although the method reaches attomole detection limit, it requires q-PCR of isolated total RNA before miRNA analysis.

The proposed method meets the sensitivity requirements with its picomolar detection limit in real samples and selective for the target miRNA. The selectivity was proven by two control studies: (1) a non-complementary miRNA, mir192 was used to show there is not either hybridization or a p19 sequestration, (2) glucose oxidase enzyme (GOX) was used instead of p19 to prove the specifity of protein to dsRNA.

2. Experimental

2.1. Apparatus and chemicals

DPV experiments were performed using the μ-AUTOLAB Type III electrochemical analysis system. A Techne-512 Thermal Cycler was used for denaturation of the stock oligonucleotides samples. Boeco Thermo-Shaker was used during solution based hybridization studies for shaking and temperature control of samples. The three electrode system consists of the pencil graphite electrode (PGE) as the working electrode, a reference electrode (Ag/AgCl) and a platinum wire as the auxiliary electrode was used in connection with the software and immersed inside a glass cell containing a specified volume of electrolyte. The volume of the electrolyte and the positions of the electrodes inside the glass cell remained constant throughout the experiments to ensure repeatibility.

A Rotring T 0.5 pencil was used as the holder for the graphite lead (Tombo HB model 0.5 mm). Electrical contact with the lead was obtained by soldering a metallic wire to the metallic part. The pencil lead was held vertically with 1.5 cm of the lead protruding outside (1 cm of which was immersed into the solution). In each experiment, the length of pencil lead was measured by the ruler, and the volumes of the blank and DNA solutions were optimized. The shape of graphite lead is cylindrical the surface area of the PGE is 1.67 mm^2.

2.2. Synthetic oligonucleotides

Synthetic oligonucleotides (as lyophilized powder) were purchased from Alpha DNA (Canada) and used without further purification. Synthetic oligonucleotides sequences are

mir21 (RNA):
5′-UAG CUU AUC AGA CUG AUG UUG A-3′
antimir 21 (RNA):
5′-UCA ACA UCA GUC UGA UAA GCU A-3′
mir21 (DNA):
5′-TAG CTT ATC AGA CTG ATG TTG A-3′
antimir21 (DNA):
5′-TCA ACA TCA GTC TGA TAA GCT A-3′
mir192 (non-complementary):
5′-CUG ACC UAU GAA UUG ACA GCC-3′

p19 was purchased from New England Bio Labs® Inc. and used without further purification. p19 siRNA Binding Protein (10 units/μl) was stored at −20 °C.

The gene of p19 siRNA binding protein is from the Carnation Italian Ringsport Virus (CIRV). Glucose oxidase (GOX) enzyme used for the purpose of controlling the biosensor selectivity. Stock solutions of RNA oligonucleotides (1000 μg/mL) were prepared in RNase-free water and were stored at −20 °C. Dilute solutions of the oligonucleotides and p19 protein were prepared daily with Tris

EDTA buffer (TEB) (10 mM Tris–HCl, 1 mM EDTA, pH: 8.00) and kept frozen. 0.5 M acetate buffer solution (ABS) containing 20 mM NaCl (pH: 4.8) was used as a buffer of measurement.

2.3. Total RNA isolation

Total RNA samples from two different cell lines PBS1 was provided by Pharmacy Department of Ege University, Izmir. Total RNA isolation was performed as previously described (Kilic et al., 2012). Briefly, total RNA was extracted from cells at 80% confluency with TRIzol reagent (Invitrogen).

2.4. Methods

Hybridization detection and mir21 (RNA)-p19 interaction methodologies were developed and optimized as described below.

2.4.1. mir21 hybridization

In the experiments, the first aim was to achieve hybridization between mir21 (RNA) (target) and antimir21 (RNA) (probe) oligonucleotides. The differences of the guanine oxidation peak currents obtained by DPV were used as the analytical signal. Hybridization procedure of the synthetic oligonucleotides consisted of the following steps; denaturation of stock RNA oligonucleotides, electrode pretreatment, hybridization, removal of unbound oligonucleotides and measurement of guanine oxidation signal with DPV.

2.4.1.1. Heat treatment of oligonucleotides

Before using synthetic oligonucleotides, they were kept at 70 °C for 5 min to prevent folding.

2.4.1.2. Electrode pretreatment

In order to obtain reproducible results by clean electrode surfaces, the electrodes were activated by application of +1.40 V for 30 s without stirring in ABS.

2.4.1.3. Hybridization

Following denaturation of RNA oligonucleotides, 6 μg/mL antimir21 and 3 μg/mL mir21 were mixed inside a vial containing TEB. The hybridization mixture was then put into the thermal shaker which was set to 65 °C and 250 rpm mixing speed and kept there for 1 h. As a control study, mir192 was used as non-complementary oligonucleotide for pseudo-hybridization in which all the parameters set were the same with real hybridization.

2.4.1.4. Immobilization

The pretreated electrodes having the 1.67 mm^2 (immersed part) were placed into 50 μL probe, hybrid and pseudo-hybrid containing solutions and kept there for 30 min. In order to prevent non-specific binding, the electrodes were rinsed by blank TEB dipping it once.

2.4.1.5. Electrochemical signal recording

The electrochemical guanine oxidation signals were measured by DPV from +0.4 to +1.4 V at 50 mV amplitude inside a glass cell containing 4 mL of ABS.

2.4.2. miRNA-p19 interaction

In these experiments, the oxidation peak current of p19 protein recorded by DPV was used as analytical signal. After hybridization, probe (antimir21 (RNA)), hybrid (antimir21 (RNA)+mir21 (RNA)) and pseudo-hybrid (antimir21 (RNA)+mir192) solutions were interacted with p19 protein. Ten μl of probe, hybrid and pseudo-hybrid solution were put into the vials separately. Ten μl of 1:20 (v/v) diluted p19 protein solutions were added into these vials which were mixed for 10 s afterwards. The interactions between the p19 protein and probe, hybrid or pseudo-hybrid proceeded for 30 min. at room temperature without any mixing.

Then, previously activated PGEs were dipped into these vials and the passive immobilization of the p19 protein–oligonucleotide complexes was achieved for 30 minutes. Next, the PGEs were rinsed in TEB for 10 s via mixing. Having rinsed, the electrodes were kept in ambient air for 5 min to dry. Finally, the electrochemical measurements were performed between the potentials +0.4 V and +1.4 V in ABS.

The electrochemical oxidation signal of the p19 protein was detected by DPV at nearly 0.80 V versus Ag/AgCl after the interaction with oligonucleotides. Label free detection of synthetic miRNA oligonucleotides based on p19 protein interaction is illustrated in Scheme 1.

3. Results and discussion

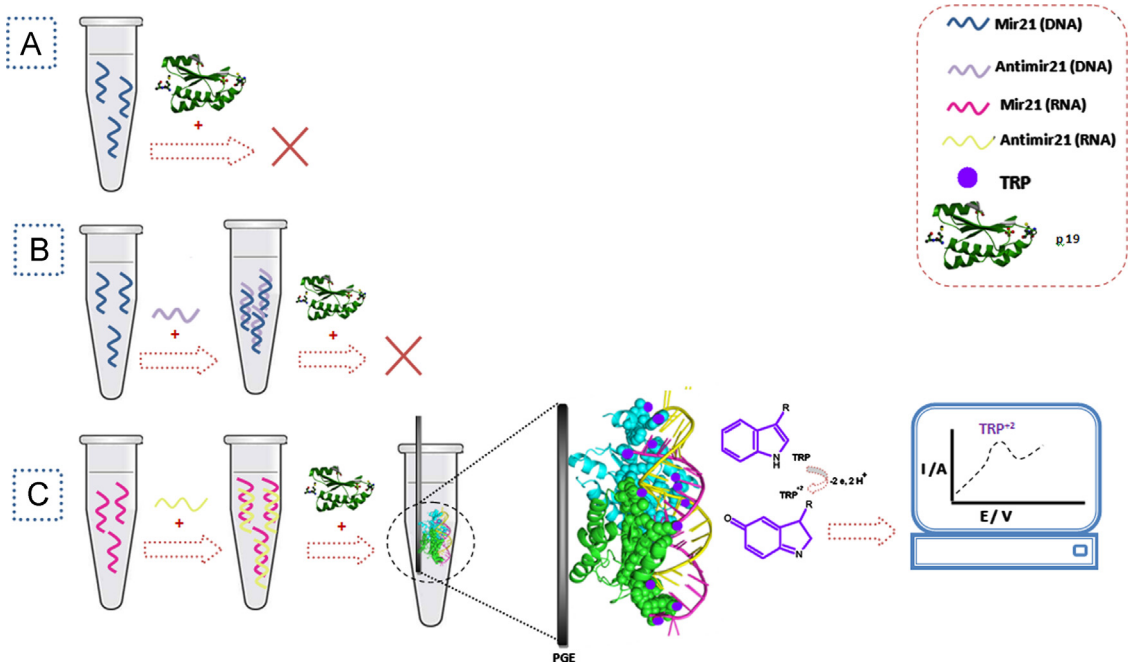
All the experiments conducted throughout the study can be divided into three main categories; optimization of solution based hybridization conditions of mir21, interaction of p19 with oligonucleotides (either DNA/RNA or single strand/double strand) and selectivity studies.

3.1. Solution based hybridization studies

As a first step of the study, conditions required for solution-based hybridization were optimized. Various parameters such as; oligonucleotides concentration, hybridization buffer, temperature, duration for electrode rinsing, speed of mixing and hybridization time was specified for the best analytical performance. All of the conditions listed in Table 1 and optimum conditions (last column, bold) were determined according to the guanine oxidation signal ratio obtained for probe an hybrid (probe/hybrid). The height of the guanine oxidation peak was used as the analytical signal with DPV.

According to the results of optimization study and the calibration plot drawn accordingly (see Supplementary fig., S1), the detection limit of the assay for $S/N=3$ was calculated as 1.6 picomole mir21 concentration in 10 μL of sample volume as outlined in reference Bard and Dekker (1990).

The achievement of hybridization was proved by comparing guanine oxidation signals recorded before and after the hybridization. In our previous miRNA study (Kilic et al., 2012), we have followed inosine based detection procedure as Lusi et al. (2009) did for their work to show the existence of hybridization. This is a well-known strategy for label free sensing but has some drawbacks for real samples. In this approach to interpret the signals of real samples are not easy due to nanoamper scale amplitudes. So, novel methods are needed for detection of miRNAs in real samples. In the first part of the study, so as to prove the existence of hybridization, we evaluated the achievement of hybridization was proved by comparing guanine oxidation signals recorded before and after the hybridization. In Fig. 1, there is a significant decrease in the peak current of guanine signal after the hybridization with anti-mir21 (RNA) and mir21 (RNA) (Fig. 1-c) compared to the signal before hybridization (Fig. 1-a). Since the hybridization was in solution phase, the guanine oxidation signal for hybrid is expected to be nearly half of both the signal recorded for anti-mir21 (RNA) (Fig. 1-a) and mir21 (RNA) (Fig. 1-b) (Meric et al., 2002). For both comparison, the hybridization seems to be successful due to the approximately 50% decrease in the oxidation



Scheme 1. Schematic presentation of electrochemical miRNA detection based on the interaction of p19 with mir21 RNA (A), hybrid of mir21 (DNA) and antimir21 (DNA) (B), hybrid of mir21 (RNA) and antimir21 (RNA) (C).

Table 1
Summary of optimization studies for hybridization detection containing probe: target concentrations, hybridization buffer, hybridization temperature, oligonucleotides attachment way, mixing speed of oligonucleotides, hybridization and rinsing time based on guanine oxidation signal ratio observed from the probe and hybrid.

Substrate	Method	Type of signal	Advantages	Drawbacks	Limit of detection	References
miRNA 122	Protein facilitated affinity capillary electrophoresis	Migration time (min)	Low assay time	Preconcentration of miRNA	0.5 fM	(Khan et al. 2011)
miRNA 122	Surface plasmon resonance	SPR intensity	Low assay time, no labeling	Needs antibody enhancement	1 fmole	(Sipova et al. 2010)
miRNA 122-a	Gel mobility shift	Amount of radioactivity	High S/N ratio, high detection limit	Needs and labeling with (γ-32)–ATP time consuming	0.1 fmole	(Lin et al. 2010)
Total RNA	Real-time quantitative PCR	Fluorescence	Speed and sensitivity, low amount of starting material	Time and reagent consuming	Not mentioned	(Benes and Castoldi 2010)
Total RNA	microarray	Fluorescence	Time efficient, easy to handle	Needs labeling	2.5 mg of total RNA	(Castoldi et al. 2006)
mir21 in MCF-7 MCF-10a Cell lines	Bioluminescent based hybridization	Light intensity	Parallel measurement high *S*/*N* ratio	Needs enzyme labeling	1 fmole	(Cissel et al. 2008)
Let 7b, Let 7c	Hybridization on interdigitated microelectrodes	Conductance	Low cost portable	No multiplexing	5.0 fmole	(Fan et al. 2007)
mir222 in Total RNA	Electrochemical detection based on paramagnetic beads and enzyme amplification	Current	Enables multiplexed analysis	Need miRNA enrichment and biotinylation prior to attachment	7 pm	(Francesca Bettazzi et al. 2013)
miR122 in human kidney and liver	Isotachophoresis and molecular beacon (MB) hybridization	Fluorescence intensity	Amplification free cheap, simple	Needs quenching	3000 copies per cell	(Persat and Sartigo 2011)

peak current for hybrid form. The reason of result is that all the guanines in the probe (either anti-mir21 or mir21) were inaccessible for oxidation after hybridization since hydrogen bond formation occurs between guanines and cytosines. On the other hand, in the case of mir192 which is designed to be used as NC, the oxidation signals almost remained the same after hybridization indicating that there is no hybridization between either antimir21 (RNA)/mir192 (RNA) (Fig. 1-d) or mir21 (RNA)/ mir192 (Fig. 1-e). From these results, it is deduced that, the hybridization was achieved only between complementary sequences and the method is selective.

Following optimization of the hybridization conditions, miRNA (RNA/DNA)–p19 protein interaction studies were performed. As indicated in other studies based on the interaction between p19 protein and miRNA, (Khan et al., 2011; Nasheri et al., 2011) p19 protein and miRNA were mixed in a buffer solution first.

Then p19 protein–miRNA complex was attached to the electrode surface by passive adsorption. Discrimination between the single and double strands in the studies where p19 protein is incorporated were done according to different types of signals; relative light units and SPR intensity (Nasheri et al., 2011), migration time (Khan et al., 2011), immunofluorescence (Calabrese and Sharp, 2006) and electrophoretic mobility (Patel et al., 2006).

In this study, the known property of p19 not to interact single stranded RNA or single/double stranded DNA but double stranded RNA in a size specific manner was the starting point and the change in the oxidation signals of tryptophan residues inside the

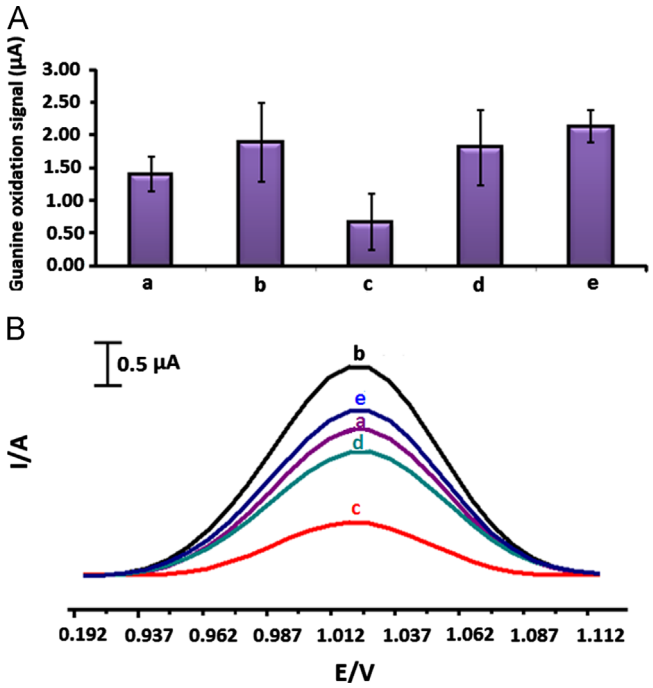


Fig. 1. Histograms with error bars (A) and differential pulse voltammograms (B) of guanine oxidation signals of synthetic RNA; anti-mir21RNA (a), mir21 RNA (b), hybrid (c), anti-mir21RNA with mir192 (d), mir21 with mir192 (e). Experimental conditions are: electrode pretreatment; +1.40 V for 60 s in 0.5 M ABS (pH:4.80 M) solution containing 20 mM NaCl, hybridization for 1 hour at 65 °C and 250 rpm, mir21 RNA—3 μg/ml, antimir21 RNA −6 μg/ml or mir192–196 μg/ml, immobilization for 30 min. Rinsing by dipping PGEs into TEA once, measurement by DPV, between +0.75 and +1.40 V in blank 0.5 M acetate buffer (pH: 4.80) solution with 20 mM NaCl.

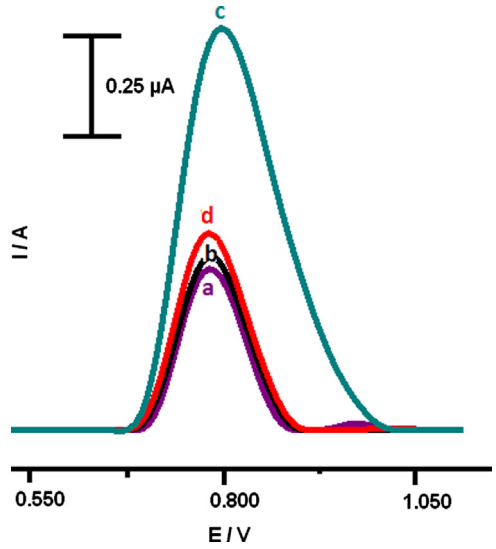


Fig. 2. p19 oxidation signals recorded before (a) and after the interactions of antimir21 (RNA) (b), hybrid (c), pseudo-hybrid (d). Experimental conditions : electrode pretreatment +1.40 V for 60 s in 0.5 M ABS (pH:4.80 M) solution containing 20 mM NaCl. Hybridization for 1 h at 65 °C and 250 rpm, mir21 RNA −3 μg/ml, antimir21 RNA −6 μg/ml or mir192–196 μg/ml, miRNA-p19 interaction; mixing of 10 μL of probe/hybrid/pseudo-hybrid solutions with 10 μL of 1:20 (v/v) diluted p19 protein for 10s, 30 min interaction of miRNA with p19 at room temperature without any mixing, immobilization of p19 – miRNA complex; 30 min., at room temperature, rinsing; 10 s via gentle mixing, drying; for 5 min, at ambient air, measurement; , scanning by DPV between +0.40 and +1.40 V in blank 0.50 M acetate buffer (pH: 4.80) solution with 20 mM NaCl.

p19 protein which is an essential amino acid and the precursor for various proteins, and also could promote the formation of peptides and proteins was used for detection of the interaction. Fig. 2 depicts the changes in the tryptophan oxidation signals before (Fig. 2-a) and after the interaction of the p19 protein with probe (Fig. 2-b), hybrid (Fig. 2c) and pseudo-hybrid (Fig. 2-d). As it is clear from the figure, the oxidation signal of the tryptophan residues in p19 protein itself observed at +0.80 V versus Ag/AgCl (Ozcan and Sahin, 2012) nearly corresponds to the signals recorded after the interaction of protein with probe and pseudo-hybrid indicating no interaction between either probe–p19 and pseudo-hybrid–p19. However, there is a dramatic increase in the tryptophan signal after the interaction of p19 with hybrid. This result is attributed to the known fact that p19 protein behaves like a molecular caliper and interacts with dsRNA in a size specific manner. The interaction between sugar–phosphate backbone or 2′ OH of duplex miRNA and p19 happens either electrostatically or through hydrogen bond formation (Ye et al., 2003). Furthermore, there are also direct and water-mediated intermolecular contacts between backbone and side chains of amino acids with the phosphate backbone and sugar 2′OH positioned towards the RNA 5′-end (Ye et al., 2003). The miRNA sugar–phosphate backbone that interacted with the p19 is distributed towards the center and ends of the miRNA duplex. The end capping tryptophans π-stacks the terminal base pairs of duplex miRNA with their imidazole ring and measure the length of miRNA by bracketing (Nasheri et al., 2011; Ye et al., 2003; Zamore, 2004). The ability of p19 to accommodate duplexes could attribute to in structural plasticity of the helices that p19 contains. In other words, drastic increase in the tryptophan oxidation signal is a result of the change in the conformation of p19 after its interaction with hybrid. The bending of 40° of siRNA having bounded by p19 so as to maximize the contact area between p19 and siRNA is an indication of such a conformational change (Cheng et al., 2008).

In order to prove the existence of the special interaction between double stranded miRNA and p19 protein, two control studies were performed. In the first one, another protein structure that contains tryptophan residues like p19 protein, glucose oxidase (GOX) enzyme was used to see whether the structure of the protein or just the tryptophan residues inside it enable such recognition. If the only cause is the existence of tryptophan amino acids, then the same results would be taken by the use of GOX since it contains tryptophan residues in its deep pocket.

On the other hand, if the position of tryptophans and conformation of the protein structure is responsible for interaction, results would be different from the ones observed with p19 protein since two protein structures are different from each other.

GOX has two FAD cofactors responsible for converting glucose to gluconic acid that is why it has been used for various glucose bio sensing platforms (Wilson and Turner, 1992). The direct electrochemistry of the FAD centers are difficult to observe due to the fact that, these moieties are surrounded by a protein shell that make them electrochemically insulated. So many approaches and materials have been investigated in 3rd generation glucose biosensors for direct electrochemistry of GOX. Here, in this study, the electrochemical signal observed at +0.80 V versus Ag/AgCl is the same signal observed for the p19 protein due to the existence of tryptophan amino acid (Liu et al., 2007; Willner and Willner, 2010).

According to the results obtained for GOX control study shown by Fig. 3A, in common with p19 protein results, there is again an oxidation signal at the same potential (at +0.80 V versus Ag/AgCl) evincing the existence of tryptophan inside GOX too. On the other hand, the interactions between GOX and antimir21 (RNA) (Fig. 3A-b), GOX and hybrid of antimir21 (RNA) with mir21 (RNA) (Fig. 3A-c), and GOX and pseudo-hybrid (Fig. 3A-d) differs from the ones obtained for p19 protein–miRNA interactions. In the case of GOX, all the signals, including the blank signal of GOX (Fig. 3A-a) have

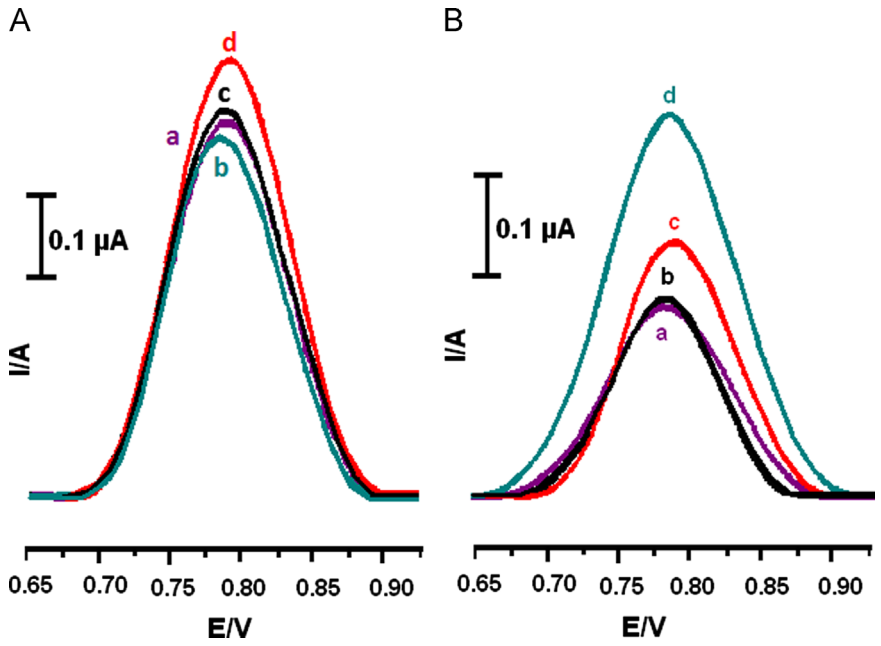


Fig. 3. Control studies for p19 protein interaction; GOX study (3A) the oxidation signals of GOX only (3A-a), and for the interactions of GOX with antimir21 (RNA) (3A-b), with hybrid of mir21 (RNA)–antimir21 (RNA) (3A-c), with pseudo-hybrid (mir21(RNA) – mir192) (3A-d) and p19 protein–DNA interaction study (3B) in which oxidation signals of p19 belong to p19 only (3Ba) and the interactions with antimir21 (DNA) (3Bb), hybrid of mir21 (RNA) with antimir21 (RNA) (3Bc), pseudo-hybrid (mir21(RNA) – mir192) (3Bd) Experimental conditions are; electrode pretreatment +1.40 V for 60 s in 0.5 M ABS (pH: 4.80) solution containing 20 mM NaCl, hybridization; 1 h at 65 C and 250 rpm, mir21 RNA −3 μg/ml, antimir21 RNA-6 μg/ml, mir192 −6 μg/ml, miRNA–GOX interaction; mixing of 10 μL of probe/hybrid/pseudo-hybrid solutions with 10 μL of 1:20 (v/v) diluted GOX enzyme for 1 0s, 30 min interaction of miRNA with GOX at room temperature without any mixing, immobilization of GOX–miRNA complex; 30 min., at room temperature, rinsing; 10 s via gentle mixing, drying for 5 min, at ambient air, measurement by using DPV, scanning between +0.40 and +1.40 V in blank 0.50 M acetate buffer (pH: 4.80) solution with 20 mM NaCl.

nearly the same current value which means there is not a specific interaction between GOX and miRNAs of any structure (ssRNA or dsRNA). If any conformational change happened on GOX, the oxidation signal of tryptophan would have been varied upon interaction with miRNAs due to the change in the accessibility of tryptophan amino acids in p19 protein. In literature, there are some reports mentioning the conformational change of GOX upon its interaction with monovalent (Ahmad et al., 2001), divalent cations (Akhtar et al., 2002) or some carbon based electrode materials (Zhao et al., 2010). In these papers, the change in the protein structure was proved by spectrophotometric methods like FT-IR, CD by the help of fluorescence property of tryptophan aminoacid. In our study, on the other hand, the electrochemical signal of tryptophan aminoacid revealed the fact that not the existence of tryptophan but the position inside the protein structure is important for sequestration of miRNAs and the caliper like property is specific for p19 protein.

In the second control study, specificity of p19 protein to different types of nucleic acids, i.e. ss/ds DNA, ss/dsRNA was tested. As it is stated in literature, the p19 protein only interacts with dsRNA rather than other types/forms of nucleic acids. According to the results shown by Fig. 2, it is already shown that there is not an interaction between ssRNA and p19 protein.

Similarly, by Fig. 3B, it is clearly seen that, tryptophan oxidation signals for probe DNA (Fig. 3B-b), hybrid DNA (mir21 (DNA)–antimir21 (DNA)) (Fig. 3B-c), and pseudo-hybrid (Fig. 3B-d) all have smaller current value than the signal of protein itself (Fig. 3B-a) as if the protein is surrounded by nucleic acids and oxidizable tryptophan residues are inaccessible for electrochemical reaction.

Also, the signals recorded for the interactions of p19 protein with hybrid, probe and pseudo-hybrid are non-distinguishable from each other. So, the specifity of p19 is not only related with the form but also the type of nucleic acid it is being interacted. The known phenomena once more proved by these experimental results that p19 protein does not interact with neither ssRNA nor ssDNA\dsDNA and only specific for dsRNA. Obtaining such distinguished results with real samples is the most important thing to be considered since the purpose of this kind of assays is to be applicable for real samples and point-of-care testing. In order to achieve this goal, the applicability of designed biosensor to real

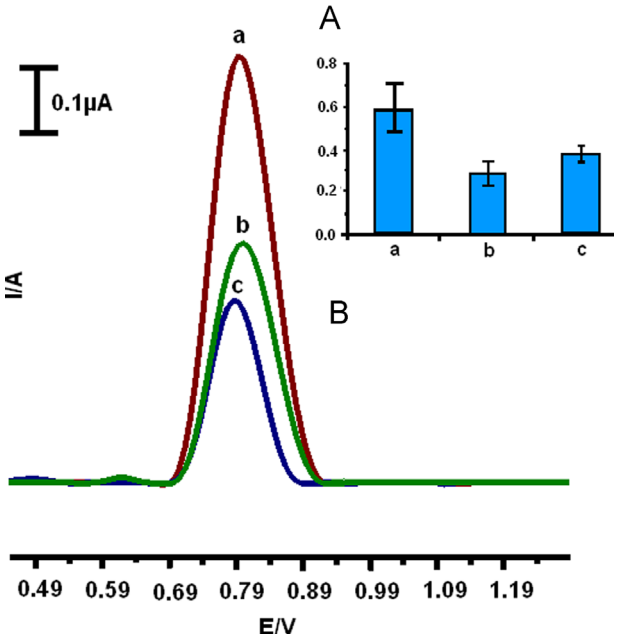


Fig. 4. Histograms with error bars (A) and differential pulse voltammograms (B) of p19 protein oxidation signals recorded for the interactions; hybrid of mir21 (inside total RNA) with synthethic antimir21 RNA oligonucleotide (a), total RNA only (b), pseudo-hybrid of mir21 (inside total RNA) with mir192 (c) experimental conditions are the same as in Fig. 2.

samples was tested by using total RNA extract of PBS1 cell line that is known to contain mir21.

Fig. 4 represents the results obtained for real sample study. As it is clear from this figure, oxidation signals of p19 protein recorded for the interaction of p19 protein with the hybrid formed between the mir21 included in total RNA and synthethic antimir21 RNA (Fig. 4-a) is higher in a considerable extent than both the signals recorded for the interactions of p19 protein with total RNA (Fig. 4-b) and pseudo-hybrid (mir21 in total RNA – mir192) (Fig. 4-c). From this result, it is deduced that, the mir21 inside total RNA was hybridized with synthetic antimir21 (RNA) and this hybrid was sequestered by p19 protein upon interaction with it. While, the protein did recognize neither the mir21 inside total RNA nor the mir21 – mir192 pseudo hybrid demonstrating once more the dsRNA recognition pattern of p19 protein for real samples too. In addition, real sample study can be assumed as another control study that shows specifity of the assay since there are various types of miRNAs inside a total RNA having different sequence than mir21 (Lu et al., 2005). If the assay is not specific for mir21, mir192 would hybridize with different miRNAs inside total RNA and generate similar signal as the one obtained for mir21-antimir21 hybrids.

4. Conclusion

Utilization of miRNAs as cancer biomarkers has become a glimmer of hope for the early diagnosis of this fatal and ever-mounting disease. Thereupon, the increasing demand for detection and identification of miRNAs resulted in various kinds of platforms designed for this purpose. Despite recent advances, current methodologies are still in their infancy since not only the detection but also quantification of miRNAs inside body fluid is needed for cancer diagnosis. By this work, a novel method that brings together the advantages of electrochemical biosensor with the size selective recognition property of p19 protein was developed. As being cost effective, sensitive and its potential to be applied in point-of-care testing, the proposed method with its advantages such as; having short assay time and high sensitivity with its picomole detection limit, direct applicability to real samples with no need of pre-amplification step prior to hybridization, no need for labeling with fluorescent or radioactive dyes, no need for post-hybridization processing like enzymatic conversion, could be adopted to various kinds of real samples and used as an alternative method for miRNA detection.

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.2013.04.011.

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