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

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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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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