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Electrochemical biosensing of microribonucleic acids using antibodies and viral proteins with affinity for ribonucleic acid duplexes

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ABSTRACT

MicroRNAs (miRNAs) are considered reliable molecular biomarkers being definitively linked to the development of relevant diseases including cardiovascular, diabetes, tissue injury and cancer. Therefore, accurate and sensitive quantification of miRNAs may result in correct disease diagnosis establishing these small noncoding RNA transcripts as valuable biomarkers. Electrochemical biosensing approaches appeared in the last 5 years for miRNAs determination based on the use of bioreceptors with affinity for RNA duplexes (viral proteins and antibodies) are ideal choices to overcome some limitations of conventional quantification strategies. In this review some of the main aspects of the interest in miRNA determination, disadvantages of conventional methodologies and unique features offered by the use of this type of versatile bioreceptors coupled to electrochemical transduction are discussed. The relevant characteristics of the reported approaches are critically commented and the research opportunities and future development trends outlined.

1. Introduction

MiRNAs are a class of small non-protein-coding single-strand (ss) ribonucleic acid molecules (RNAs) of 19 to 25 nucleotides (nts) with 2 nt 3′ overhanging ends and 5′ phosphate groups which play important regulatory roles in various physiological (cell development, differentiation, metabolism, immune system and apoptosis) and pathological (oncogenesis) processes [1]. Accumulated evidences have indicated that aberrant expression of miRNAs is found to accompany many illnesses, such as diabetes, cancer, heart diseases, tissue injury after stroke and neurodegenerative disorders. Accordingly, miRNAs can be considered as important and minimally invasive informative targets for both early and reliable diagnostic and therapeutic applications [2–7]. MiRNAs found in blood serum, plasma, urine, saliva, and other body fluids can be either up or down regulated in these diseases and figuring out their regulation pattern is of great importance since it could provide information about their potential roles in initiation and progression of these illnesses [8]. Moreover, miRNAs possess ideal biomarker's characteristics such as specificity for the particular disease or pathology, reliable indication of the disease before appearing clinical symptoms, sensitivity to changes in the pathology progression or therapeutic response, and feasibility to be sampled by a relatively non-invasive method [9,10]. Consequently, analytical methods able to detect and accurately quantify in a reliable manner the expression levels of one, or more interesting, a set of miRNAs are extremely desirable in biomedical research and clinical diagnosis and expected to have a tremendous impact on healthcare [4,11].

Although many efforts have been made in the detection of miRNAs, their short sequence length (most miRNAs are approximately 21-22 nts, i.e. the length of a standard PCR primer) high sequence similarity among family members (miRNAs of the same family may differ by only one base), low abundance (approximately 0.01% of the total RNA typically extracted from a sample) wide concentration range (the miRNAs expression level may vary in samples in more than 4 orders of magnitude) and susceptibility to degradation can be mentioned as factors which make difficult the accurate detection and quantification of miRNAs using conventional RNA analytical techniques [1,2,12–16]. Current methods for miRNA detection include quantitative reverse transcription polymerase chain reaction (qRT-PCR), microarray-based detection, capillary electrophoresis, and northern blot analysis. These methods provide a high sensitivity but they meet only partially the requirements for routine detection and have some disadvantages such as providing only a limited degree of qualitative data, high assay costs, time consuming steps, and low detection accuracy [5,17,18]. In this context, electrochemical biosensors hold

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great promise as one of the most appealing alternatives to conventional methodologies for miRNA determination [19–32]. Relevant functional characteristics such as fast response, affordable cost, ease of operation, automation and *on-site* analysis meet adequately the requirements needed for routine detection [11,19,32–38].

2. Electrochemical biosensors for miRNAs determination using viral proteins and antibodies as bioreceptors

Although a noticeable number of electrochemical biosensors have been reported so far for miRNAs determination [21–32], most of them require the use of multiple reagents and involve complex and time-consuming working protocols including amplification strategies.

Particularly attractive and simple strategies are those involving antibodies and viral proteins as versatile bioreceptors specifically directed to RNA/DNA and RNA/RNA hybrids, respectively. Some interesting reports for miRNAs determination have appeared in the last 5 years using these antibodies [3,11,33,38,39–41], and viral proteins [2,5,7,14,17,34,42,43]. These specific and versatile bioreceptors exhibit unique binding properties allowing detecting mature miRNAs transcripts with high selectively. Apart for their selectivity towards a particular type of duplexes (DNA/RNA) other key advantage of the use of specific antibodies for miRNA detection is the fact that there are versatile recognition elements for any target miRNA which is particularly valuable for the development of multiplexed detection platforms.

Previous strategies utilizing monoclonal and polyclonal antibodies for recognition of DNA/RNA hybrids, such as antipoly(A)–poly-(dT) and anti-S9.6 [33], and double-stranded (ds)RNA have been proposed in hybridization based assays for nucleic acid detection [11]. It has been reported that S9.6 (also known as anti-DNA/RNA antibody) exhibits high specificity and affinity for DNA/RNA hybrids, and it does not cross-react with ssDNAs or dsDNAs and RNAs [11,12]. It has been shown that a single DNA/RNA heteroduplex can be bound by multiple S9.6 antibodies and that the S9.6 binding epitope is of the order of 6 base pairs in size [13].

A large number of viral proteins involved in DNA replication, damage control, repair, and gene expression are capable of binding DNA and RNA with different affinities and sequence specificities [44]. These viral proteins can interact with small RNAs such as miRNAs and siRNAs preventing viral RNA degradation and allowing the establishment and maintenance of a systemic infection [45,46]. Some examples of these viral proteins are the ssDNA binding protein (SSB) [44], the beet yellow virus p21 [47], the Tombusviral p19 and the Tospoviral RNA Silencing Suppressor Proteins [48].

Among the Tombusviral p19 proteins, the Carnation Italian Ringspot virus (CIRV) is, together with the tomato bushy stunt virus (TBSV) variant, one the best characterized structurally and functionally [49,50] and, probably because of its commercial availability, the only one used in the electrochemical biosensing so far. CIRV p19 protein (19 kDa) is a RNA silencing suppressor which behaves as a molecular caliper to sequester selectively 19-23 base pair dsRNA with nanomolar affinity in a size dependent and sequence independent manner instead of binding to ssRNA, rRNA, ssDNA, dsDNA or mRNA [7,51–53]. Accordingly, binding of p19 to dsRNA can be exploited for recognition of miRNAs after hybridization with specific RNA probes [4]. The binding between p19 and dsRNAs occurs through electrostatic and hydrogen-bonding interactions between the β-sheet formed by the p19 homodimer and the sugar–phosphate backbone of dsRNA, thereby making the binding sequence independent of the RNA substrate [2]. Previous studies demonstrated that the p19 protein exhibits a high affinity for 21 nts RNA duplexes containing a 2-base overhang and 5′ phosphate groups which interact with the tryptophan residues on the endcapping helices of the p19 dimer for stabilization [50,54–56]. The affinity of this viral protein for ds-RNA duplexes decreases significantly as the duplex region is lengthened or shortened. Actually it was showed that 320 and 75-fold lower affinities occurred for 19 and 26-mer RNA duplexes [53].

It is important to remark that, unlike with sequence-specific RT-PCR primers, the biosensing approaches using viral proteins and antibodies as bioreceptors are readily translated to the interrogation of any target miRNA simply by adding the complementary probe to the target miRNA to form the corresponding DNA/miRNA or RNA/miRNA duplex. Therefore, these methodologies merge the selectivity of target miRNA/complementary probe hybridization with the unique specificity of these bioreceptors for the particular duplex. Moreover, unlike what occurs with p19 protein which acts like a molecular caliper clasping the ds-RNA, the small size of S9.6 antibodies epitope allows multiple antibodies to bind single DNA/miRNA heteroduplexes which in turn permits enhanced signal amplification.

Taking advantage of the unique properties of viral proteins and antibodies as bioreceptors, very attractive and promising strategies for electrochemical miRNAs biosensing have been developed. Therefore, the following subsections will be devoted to the brief description of the fundamentals of the different developed strategies. Table 1 highlights the type of bioreceptor used, the target miRNA and the electrochemical technique employed for transduction. The basis of each methodology, the analytical characteristics and main advantages and limitations will be briefly discussed in the following sections, together with future prospects and challenges to face in this field.

2.1. Viral proteins

By pairing up the advantages of electrochemical sensors with the unique binding properties of CIRV p19 protein to small dsRNAs, some interesting approaches for the determination of miRNAs, using both label-free and labeling strategies have been described recently.

A label-free electrochemical biosensor for the determination of miRNA-21 was reported by Kilic et al. using passive immobilization of the p19-dsRNA bioconjugates onto activated pencil graphite electrodes (PGEs) [7]. The analytical readout consisted of measuring the differential pulse voltammetric (DPV) oxidation signal of the p19 protein tryptophan residues (Fig. 1). This voltammetric miRNA assay allowed achieving a LOD of 1.6 picomole of the target miRNA in 10 μL. The applicability of this biosensor was demonstrated by determining miRNA-21 in total RNA (RNA_t) extracted from PBS1 cell line.

Labib et al. [2] designed a three-mode electrochemical biosensor able to detect 5 different miRNAs (miRNA-21, miRNA-32, miRNA-122, miRNA-141, and miRNA-200 reported as colorectal, prostate, liver, colon and ovarian tumors biomarkers, respectively) using self-assembly of a thiolated RNA onto a gold nanoparticles-modified screen-printed carbon electrode (AuNPs-SPCE). This approach was claimed to detect one or multiple miRNAs using three detection modalities involving direct hybridization, p19 binding and displacement. In all cases electrochemical signals corresponded to the amplified current density, measured by square wave voltammetry (SWV) at the modified electrodes in the presence of $K_3[Fe(CN)_6]$ and $[Ru(NH_3)_6]Cl_3$, and corresponding to the reduction of the positively charged Ru^{3+} complex electrostatically adsorbed on negatively charged RNAs and the recycling of the generated Ru^{2+} by the Fe(III) in solution [36]. The hybridization of the target miRNA to its complementary immobilized probe caused an increase in the electrochemical signal due