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An electrochemical biosensor for highly sensitive determination of microRNA based on enzymatic and molecular beacon mediated strand displacement amplification

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ABSTRACT

In this study, a simple electrochemical biosensor has been established for highly sensitive and specific detection of target microRNA using molecular beacon mediated strand displacement amplification and enzymatic amplification. In the presence of target microRNA, the specific hybridization of microRNA with corresponding domain opens the hairpin structure of a molecular beacon (MB), which triggers strand displacement amplification (SDA) to release nicking DNA triggers. The part of DNA triggers binds to the capture probes immobilized on the gold electrode. Another part of DNA triggers hybridizes with the biotinylated detection probes. The electrochemical signal is obtained by using streptavidin linked to alkaline phosphatase (ST-AP) toward the synthetic enzyme substrate α-naphthyl phosphate (α-NP). Under the optimal conditions, the established biosensor shows high sensitivity and selectivity in a dynamic response range from 50 pM to 10 nM with a detection limit as low as 40 pM (S/N = 3), and good specificity and acceptable reproducibility are achieved for the miRNA-222 detection. This simple and highly efficient biosensor can provide a sensing platform for clinical molecular diagnostics and biomedical research.

1. Introduction

MicroRNAs (miRNAs) are short nucleotide RNA molecules (21–24 nt), which can regulate the expression of target genes in cell proliferation, differentiation, and tumorigenesis [1,2]. Miscellaneous evidences demonstrate that the unnatural expression of miRNAs is frequently dysregulated in the development of a variety of cancers and other diseases [3,4]. In addition, miRNA expression patterns in cancers appear to be tissue-specific, which makes miRNAs be promising biomarkers [5]. Thus, quantitative analysis of miRNAs is vital for not only better understanding their effects in cancers and other diseases but also further confirming their functions in clinical diagnosis and biomedical research.

MiRNAs have short lengths, low abundance in total RNA samples, susceptibility to degradation, and sequence similarity among family members [6], weakening the traditional and standard methods for miRNA analysis. The traditional methods for miRNA analysis are Northern blotting [7,8], microarray analysis [9,10] and real-time quantitative polymerase chain reaction (qRT-PCR) [11,12]. Northern blotting is widely utilized to visualize specific analysis of miRNAs, but it is very time-consuming, sample-consuming, and semi-quantitative with low sensitivity and throughput. The microarray can achieve multiple miRNA analysis, but it frequently suffers from large sample size, unrealistically lengthy hybridization time, and excessive variations between protocols, which limit its wide application [13]. qRT-PCR has been developed for sensitive detection of miRNAs. However, it requires complex and tedious steps for miRNAs isolation and purification, generating false positive. Recently, biosensor methods for miRNA detection have gained conspicuous attention, such as colorimetry [14], fluorescence [15], surface plasmon resonance [16], and electrochemistry [17]. Among these methods, electrochemical biosensor has been attracted increasing attention owing to simplicity, low cost, high sensitivity, and easy of miniaturization [18–20].

In recent years, to further enhance the sensitivity of biosensing detection, different amplification strategies based on isothermal exponential amplification reaction (EXPAR) have been aroused increasing interest in the field of biosensors [21–23], such as strand displacement amplification (SDA) [24,25], rolling circle amplification (RCA) [26], loop-mediated amplification (LAMP) [27], and enzymatic repairing amplification (ERA) [28,29]. Among the isothermal amplification technologies, strand displacement amplification (SDA) has been attracted more and more attention due to its fast, efficient, and no requirement special equipment [30,31]. Moreover, SDA can provide exponential

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amplification of a trace of DNA or RNA, which is an important step for nucleic acid detection [32–34]. Therefore, SDA can be utilized as signal amplification strategy for highly sensitive and simple determination of miRNA.

Herein, we reported a simple and sensitive electrochemical biosensor for detection of miRNA based on molecular beacon mediated strand displacement amplification and enzymatic amplification. In this assay, we designed a molecular beacon with the hairpin structure that unfolded through the target miRNA. The inherent properties of MB mediated strand displacement amplification and enzymatic amplification would ensure high sensitivity and specificity of the designed biosensing strategy. The developed strategy exhibited excellent analytical performance toward miRNA detection, which might provide a promising sensing platform for the bioanalysis and clinical molecular diagnostics.

2. Experimental

2.1. Reagents and materials

Klenow fragment (3′–5′ exo) and Nb.BbvCI were purchased from New England Biolabs (Beijing, China). Diethylpyrocarbonate (DEPC), bovine serum albumin (BSA), salmon sperm DNA, and deoxynucleotide solution mixture (dNTPs) were purchased from Sangon Inc. (Shanghai, China). 6-Mercapto-1-hexanol (MCH), streptavidin-alkaline phosphatase (ST-AP), and α-naphthyl phosphate (α-NP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MiRNAs and RNase inhibitor were obtained from Takara (Dalian, China). All other reagents were of analytical grade. All aqueous solutions were prepared using Millipore-Q water (≥18MΩ, Milli-Q, Millipore). Hybridization buffer (pH 7.4) contained 0.3 M NaCl and 0.03 M sodium citrate. Tris–HCl buffer as washing buffer (pH 7.4) contained 20 mM Tris–HCl, 100 mM NaCl, 5 mM $MgCl_2$ and 0.05% Tween-20. Diethanolamine (DEA) buffer (pH 9.6) contained 0.1 M DEA, 1 mM $MgCl_2$, and 100 mM KCl. NEBuffer 2 contained 10 mM pH 7.9 Tris–HCl, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. CutSmart™ Buffer contained 20 mM pH 7.9 Trisacetate, 500 mM potassium acetate, 10 mM magnesium acetate, and 100 μg mL^{-1} BSA. Trisethylenediaminetetraacetic acid (TE) buffer (pH 8.0) contained 10 mM Tris–HCl, 1 mM EDTA.

All oligonucleotides were synthesized and purified by Sangon Inc. (Shanghai, China), and the base sequences are listed in Table 1. All oligonucleotides were dissolved in TE buffer and stored at −20 °C, which were diluted in appropriate buffer prior to use.

2.2. Apparatus

Electrochemical signal was monitored by CHI660D electrochemical workstation (Shanghai Chenhua Instruments Co. Ltd., China) with a conventional three-electrode system composed of a 3-mm diameter gold electrode as a working electrode, Ag/AgCl electrode as reference, and platinum wire as an auxiliary. Differential pulse voltammetry (DPV), square wave voltammetry (SWV), and electrochemical impedance spectroscopy (EIS) were carried out at room temperature. The gel electrophoresis was performed on the DYY-6C electrophoresis analyzer (Liuyi Instrument Company, China) and imaged on Bio-rad ChemDoc XRS (Bio-Rad, USA).

Table 1
Oligonucleotides used in the present work.

Nucleic acid[a]	Sequence (5′–3′)
miRNA-222	AGCUACAUCUGGCUACUGGGUCUC
miRNA-21	UAGCUUAUCAGACUGAUGUUGA
SM	AGCUACAUCUGGCUACUGGGUUUC
DM	AGCUACAUCUGGCUACUGGUUUUC
NC	UCCAUCUACACCCAUCACCCACAC
MB	TGGAGTGTGACAATGGTGTTTGTTCCTCAGCAGC TACATGAGACCCAGTAGCCAGATGTAGCTTTT
Capture probe	TTTTTTTGGAGTGTGACAATG-SH
Detection probe	Biotin-GTGTTTGTTCCTCATTTTTT

[a] SM, single-base mismatched miRNA-222; DM, double-base mismatched miRNA-222; NC, non-complementary mismatched miRNA-222; MB, molecular beacon with C6 SPACER protected 3′-end.

2.3. Preparation of the electrochemical biosensor

The bare gold electrode was polished with 0.05 μm alumina slurries and ultrasonically treated in ultrapure water for a few minutes, followed by soaking in piranha solution (H_2SO_4:H_2O_2 = 3:1) for 10 min to eliminate other substances. The pretreated gold electrode was rinsed with ultrapure water and allowed to dry at room temperature. 10 μL of 1 μM thiolated capture probes in TE buffer was dropped on the pretreated gold electrode surface and incubated overnight at 4 °C. After being washed with the washing buffer, the electrode was immersed into 10 μL of 1 mM MCH for 1 h to obtain well-aligned DNA monolayer which would occupy the left bare sites. The electrode was further rinsed with the washing buffer and treated in 2% BSA and 125 μg mL^{-1} salmon sperm DNA for 30 min to block the nonspecific binding sites on its surface.

2.4. Assay protocol for target miRNA

The strand displacement amplification reaction was initiated by adding 3 μL target miRNA with different concentrations, 6 μL of 10 nM MB probes, 0.6 μL of 0.1 UμL^{-1} Klenow fragment, 0.6 μL of 0.2 UμL^{-1} Nb.BbvCI, 0.7 μL of 250 μM dNTP, 0.9 μL of 1.2 UμL^{-1} RNase inhibitor, 3 μL NEBuffer 2, 3 μL CutSmart™ Buffer and 10.2 μL DEPC-treated water. Then the mixtures of SDA reaction were incubated at 37 °C for 1.5 h. After the strand displacement amplification reaction, Klenow fragment (3′–5′exo) and Nb.BbvCI were inactivated by heating the reaction mixture at 65 °C for 10 min. The resulting mixture could be directly used to following experiment or stored at −20 °C. Next, DNA triggers hybridized with the capture probes immobilized on the gold electrode for 1 h at 4 °C. After being washed with the washing buffer, biotinylated detection probes were then dropped on the biosensor and hybridized with another part of DNA triggers for 0.5 h at 4 °C. Washed by DEA buffer, the fabricated biosensor reacted with 10 μL of 1.25 μg mL^{-1} ST-AP at 4 °C for 30 min, and washed thoroughly with DEA buffer. The DPV measurement was performed in DEA buffer containing 1.0 mg mL^{-1} of α-NP substrate with modulation time of 0.05 s, interval time of 0.017 s, step potential of 5 mV, modulation amplitude of 70 mV and potential scan from 0.0 to +0.6 V.

3. Results and discussion

3.1. Design of the electrochemical biosensor

The principle for miRNA detection is illustrated in Scheme 1. The MB template consists of three domains: miRNA-binding domain, recognition domain for nicking by Nb.BbvCI, and amplification domain for producing DNA triggers. In the presence of target miRNA, the specific hybridization of miRNA with corresponding domain opens the MB template, which leads to a part duplex. Then, along the template the target miRNA is extended to form a complete duplex by Klenow fragment and dNTPs. Subsequently, the nicking enzyme can specifically recognize the duplex nicking site, cleaving the upper extended DNA strand and exposing a new replication site for polymerase. Thus, a number of DNA triggers are produced through the continuously extension, cleavage, and strand displacement amplification (SDA), resulting in the great amplification of detection signal. One part of DNA triggers can hybridize with capture probe immobilized on the electrode and the other part of DNA triggers hybridize with biotinylated detection probe, which can link to ST-ALP and produce enzymatic electrochemical signal for quantitative detection of miRNA.