Biosensors and Bioelectronics 79 (2016) 79–85

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Electrochemical biosensor for microRNA detection based on poly (U) polymerase mediated isothermal signal amplification

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ARTICLE INFO

Article history:
Received 4 September 2015
Received in revised form
3 December 2015
Accepted 7 December 2015
Available online 9 December 2015

Keywords:
MicroRNA detection
Electrochemical biosensors
Poly(U) polymerase
Phytohormone
Rice seedlings

ABSTRACT

MicroRNAs play crucial role in post-transcriptional regulation for gene expression in animals, plants, and viruses. For the better understanding of microRNA and its functions, it is very important to develop effectively analytical method for microRNA detection. Herein, a novel electrochemical biosensor was fabricated for sensitive and selective detection of microRNA based on poly(U) polymerase mediated isothermal signal amplification, where poly(U) polymerase can catalyze the template independent addition of UMP from UTP to the 3' end of RNA. Using this activity, the target microRNA can be successfully labeled with biotin conjugated UMPs at its 3′-end using biotin conjugated UTP (biotin-UTP) as donor. Then, the avidin conjugated alkaline phosphatase can be further captured to the 3′-end of the target microRNA based on the specific interaction between biotin and avidin. Finally, under the catalytic activity of alkaline phosphatase, the substrate of *p*-nitrophenyl phosphate disodium salt hexahydrate can be hydrolyzed to produce 4-nitrophenol. According to the relationship between the electrochemical signal of *p*-nitrophenol and the concentration of microRNA-319a, the content of microRNA-319a can be detected. This signal amplification method is simple and sensitive. The developed method can detect as low as 1.7 fM microRNA and produce precise and accurate linear dynamic range from 10 to 1000 fM. The fabricated biosensor was further applied to detect the expression level change of microRNA-319a in rice seedlings after incubation with five kinds of different phytohormones.

1. Introduction

MicroRNAs are short, endogenous and noncoding RNA, which have been found in animals, plants and viruses, and play crucial roles in gene regulation, cell proliferation, cell apoptosis (Winter et al., 2009). It has been reported that the expression level change of microRNAs are not only associated with various human diseases, especially cancers, but also related to the signal transduction of phytohormone in plant growth and development process (Calin and Croce, 2006; Liu and Chen, 2009). In order to further understand the functions of microRNA in detail, it is necessary to develop sensitive and selective method for microRNA detection.

The traditional methods for microRNAs detection are northern blotting (Pall et al., 2007), microarray (Li and Ruan, 2009) and qRT-PCR (Kroh et al., 2010). Though these methods showed great applicability for microRNA detection, there are still some weaknesses. Northern blotting needs large amount of detection sample and time-consuming detection process. And this method can not quantitative detect microRNA with high sensitivity and specificity. Microarray requires high consumption and complicated detection process. Moreover, the microarray technique also suffers from the low distinguishing ability for microRNA precursor and matured microRNA. For qRT-PCR, the reverse transcription from miRNA to cDNA is rather difficult because of the small size of miRNA, which requires to design sophisticated sequences.

In order to overcome the disadvantages of the above methods, many other analytical techniques, including electrochemistry (Bartosik et al., 2014; Liu et al., 2015; Miao et al., 2015; Wang et al., 2014a; Wu et al., 2013; Yang et al., 2014a; Yu et al., 2014; Zhu et al., 2014), fluorescence (Dong et al., 2014; Ge et al., 2014a; Ho et al., 2014; Xi et al. 2014; Xu et al., 2014), colorimetry (Park and Yeo, 2014; Shen et al., 2013), UV–visible spectroscopy (Deng et al., 2014), photoelectrochemistry (Cao et al., 2014; Wang et al., 2014b; Yin et al., 2014), chemiluminiscence (Bi et al., 2015; Zhang et al., 2015b), electrochemiluminescence (Cheng et al., 2014; Hao et al., 2014; Zhang et al., 2015a), surface plasmon resonance (Ding et al., 2015; Vaisocherová et al., 2015; Zhang et al., 2013), have been

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http://dx.doi.org/10.1016/j.bios.2015.12.009
0956-5663/

developed for sensitive detection of microRNA. For obtaining high detection sensitivity, the appropriate and effective signal amplification technique is required. Among various signal amplification strategies, isothermal amplification approaches have attracted more and more attentions in the field of microRNA detection due to high signal amplification capability, such as rolling circle amplification (Ge et al., 2014a; Xu et al., 2014; Zhang et al., 2015a), hybridization chain reaction amplification (Ge et al., 2014b; Liu et al., 2015; Zhang et al., 2014), isothermal exponential amplification reaction (Jia et al., 2010; Yu et al., 2014), ligase chain amplification reaction (Yan et al., 2010; Zhu et al., 2014) and loop-meditated isothermal amplification (Li et al., 2011). All the above amplification strategies require specific nucleic acid sequence and complicated amplification process, which might limit the application of the above mentioned methods. In this regard, it still needs to develop efficient microRNA detection techniques with simple signal amplification strategy.

Recently, several reports have drawn our attentions on DNA detection using a kind of unique *DNA polymerase* named *terminal deoxynucleotidyl transferase* (TdT) (Lin et al., 2015; Wan et al., 2014a), where the TDT is a template independent polymerase that catalyzes the addition of deoxynucleotides to the 3′ hydroxyl terminus of DNA molecules. For instance, Wan et al. introduced TDT into the fabrication of electrochemical DNA biosensor (Wan et al., 2013). After probe DNA hybridized with target DNA, the TDT-mediated extension reaction of target DNA at its 3′-terminal was preformed, where biotin conjugated deoxyadenosine triphosphates (Biotin-dATPs) were added into the mixture of dNTP. Thus, the extension DNA chain was labeled with biotin, which resulted in the further capture of avidin conjugated horseradish peroxidase (HRP) on the electrode surface through the specific interaction between biotin and avidin. Based on the catalysis effect of HRP, the target DNA was detected. Yang et al. reported a template-free signal amplification strategy for highly sensitive and sequence-specific DNA detection based on *in situ* grown DNA nanotail (IGT) to capture the electrochemical signal probe of $[Ru(NH_3)_6]^{3+}$, where the IGT was produced under the catalysis of TDT in the presence of dNTP (Yang et al., 2014b). By introduction of TdT to this sensor design, both the sensitivity and selectivity have been significantly enhanced with the low detection limit of 20 fM for 22 nt DNA and the linear range of 0.1–1000 pM. Hu et al. (2015) developed a novel label-free amplified multifunctional strategy of dendritic electrochemical DNA sensor based on TdT mediated DNA extension and DNA dendritic structure signal amplification. The fabricated biosensor presented excellent detection sensitivity with low detection limit of 1 fM. The signal amplification strategy based on TDT shows several advantages, such as easy operation, multiplex labeling, high detection sensitivity and reaction carried out under isothermal condition.

Inspiring by these investigations, a simple and sensitive electrochemical method was developed for microRNA detection based on poly(U) polymerase mediated isothermal signal amplification, where poly(U) polymerase can catalyze the template independent addition of uridine monophosphate (UMP) from uridine triphosphate (UTP) to the 3′-terminal of RNA (Lehrbach et al., 2009; Rissland and Norbury, 2008). In this work, biotin conjugated UTP (biotin-UTP) was selected as UMP donor to form poly(U)-biotin tail at the 3′-terminal of hybridized microRNA. Based on the biotin-avidin bridge, a large amount of avidin conjugated alkaline phosphatase (avidin-ALP) was further captured to catalyze the hydrolysis reaction of *p*-nitrophenyl phosphate disodium salt hexahydrate (PNPP). Based on the electrochemical signal of *p*-nitrophenol (PNP), the microRNA detection can be achieved. The effect of phytohormone on the expression level of microRNA in rice seedlings was also investigated.

2. Experimental

2.1. 1 Materials and methods

Biotin-UTP was purchased from Biocompare (USA). Gibberellin, abscisic acid, 6-benzyladenine, chloroauric acid and PNPP was obtained from Shanghai Dibo Chemical Technology Co., Ltd. (China). Poly(U) polymerase was provided by New England Biolabs (USA). Diethypyrocarbonate (DEPC) was supplied by Solarbio (China). Tris(hydroxymethyl)aminomethane (Tris), tris(2-carboxyethyl)phosphine hydrochloride, 3-indoleacetic acid and 3-indolepropionic acid were purchased from Aladdin (Shanghai, China). 3-Mercaptopropionic acid (MPA) was obtained from Alfa Aesar (UK). HPLC-purified microRNAs were purchased from Takara Biotechnology Co. Ltd. (Dalian, China). DNA probe and avidin-ALP was supplied by Sangon Biotech (Shanghai) Co.,Ltd. The oligonucleotides sequences were shown as follows. Probe DNA, 5′-SH-$(CH_2)_6$-GGGAGCACCCTTCAGTCCAA-3′; target microRNA-319a, 5′-UUGGACUGAAGGGUGCUCCC-3'; single-base mismatched microRNA, 5′-UUGGACUG***U***AGGGUGCUCCC-3′; three-base mismatched microRNA, 5′-UU***C***GACUGA***U***GGGUGCUC***G***C-3′; non-complementary microRNA, 5′-ACCAGCCAGGUCCAAUCUAA-3′. Biotin labeled microRNA-319a, 5′-biotin-UUGGACUGAAGGGUGCUCCC-3′. The nucleotide mismatches were indicated as italic and bold letters. Synthetic DNA and microRNA sequences were dissolved in TE buffer (pH 8.0) according to the manufacturer's recommendations, and stored at −20 °C and −80 °C, separately. All other reagents were analytically pure grade.

The buffer solutions used in this work were listed as follows. DNA immobilization buffer, 10 mM Tris–HCl, 1.0 mM EDTA, 1.0 M NaCl and 1.0 mM TCEP (pH 7.4). MicroRNA hybridization buffer, 1 × SSC (0.15 M sodium chloride and 15 mM sodium citrate). Poly (U) polymerase reaction buffer, 50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT (pH 7.9). Electrochemical detection buffer for differential pulse voltammetry (DPV), 10 mM Tris–HCl containing 0.6 mM PNPP and 0.1 mM $MgCl_2$ (pH 9.8). Washing buffer, 10 mM Tris–HCl containing 50 mM NaCl (pH 7.4). Electrochemical detection buffer for electrochemical impedance spectrum (EIS), 10 mM Tris–HCl containing 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$ and 0.1 M KCl (pH 7.4).

2.2. Sample treatment and analysis

The rice (it was supplied by Beijing Normal University, The name of rice variety is japonica rice Oryza sativa cv. Hwa-young) was cultivated and treated according to our previous work except that five kinds of plant hormones were used in this work (Wang et al., 2015). In brief, mature rice seeds were soaked and embedded in gauze for about 10 days at room temperature for germination with deionized water. After these seeds germinated, they were transferred to culture dishes and incubated at room temperature for further experiments on the effect of phytohormone on the expression level of microRNA-319a. For investigating the concentration effect, 50 mg/L of gibberellin, abscisic acid, 6-benzyladenine, 3-indoleacetic acid and 3-indolepropionic acid were sprinkled on three groups of the leaves of rice seedlings as water source, respectively. And then these rice seedlings were cultured for 24 h. For control, one group rice seedling was cultured with distilled deionized water as water source. Finally, the seedlings with different treatment were harvested for RNA extraction. The extraction procedure was performed by using RNA extraction kit according to the manufacturer′s recommended protocol (Invitrogen, USA).

In order to analyze the expression level of microRNA-319a in the extracted total RNA and confirm the effect of five kinds of phytohormones on microRNA-319a expression, the probe DNA on