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An electrochemical microRNAs biosensor with the signal amplification of alkaline phosphatase and electrochemical–chemical–chemical redox cycling

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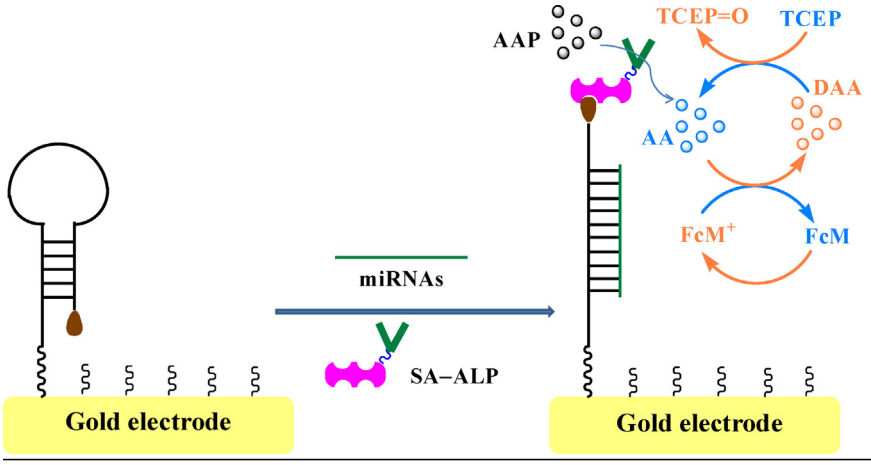
HIGHLIGHTS

- We reported a simple and sensitive electrochemical method for microRNAs detection.
- The signal was amplified by single enzyme (alkaline phosphatase).
- Electrochemical–chemical–chemical redox cycling was employed in the detection system.
- The detection limit of this method was 0.2 fM.
- The work should be valuable for designing new types of electrochemical biosensors.

GRAPHICAL ABSTRACT



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ABSTRACT

MicroRNAs (MiRNAs) have been regarded as clinically important biomarkers and drug discovery targets. In this work, we reported a simple and ultrasensitive electrochemical method for miRNAs detection based on single enzyme amplification and electrochemical–chemical–chemical (ECC) redox cycling. Specifically, upon contact with the target miRNAs, the hairpin structure of biotinylated DNA immobilized on gold electrode was destroyed and the biotin group in DNA was forced away from the electrode surface, allowing for the coupling of streptavidin-conjugated alkaline phosphatase (SA-ALP). Then, ascorbic acid (AA, the enzymatic product of ALP) triggered the ECC redox cycling with ferrocene methanol (FcM) and tris(2-carboxyethyl)phosphine (TCEP) as the redox mediator and the chemical reducing reagent, respectively. The method was more sensitive than that with horseradish peroxidase (HRP) or glucose oxidase (GOx) triggered recycling since one ALP molecule captured by one target miRNA molecule promoted the production of thousands of AA. Analytical merits (*e.g.*, detection limit, dynamic range, specificity, regeneration and reproducibility) were evaluated. The feasibility of the method for analysis of miRNA-21 in human serum has also been demonstrated.

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1. Introduction

MicroRNAs (MiRNAs) are a class of small endogenous 21–25 nucleotide-long noncoding RNAs that play important functions in numbers of biological processes, including developmental regulation, proliferation, differentiation, cardiogenesis, and epigenetic inheritance. There is a growing evidence of links between miRNAs expression and the onset of cancer (prostate, breast, colon, lung, *etc.*) and other diseases (diabetes, heart diseases, *etc.*) [1,2]. MiRNAs-based regulation is also implicated in disease etiology, and some tumor-related miRNAs have been detected in serum [3,4]. Thus, miRNAs have been regarded as clinically important biomarkers and drug discovery targets [5,6].

At present, miRNAs are predominantly analyzed using real time polymerase chain reaction (RT-PCR) and Northern blotting with optical detection. Only recently, new methods have emerged which are not associated with standard RT-PCR and Northern blotting. The novel methods based on various detection ways reduce procedural complexity and expenses, including colorimetry [7–10], fluorescence [11–16], electrochemistry [17–25], capillary electrophoresis [26,27], surface plasmon resonance [28], surface enhanced Raman scattering [29,30], electrochemiluminescence [31] and bioluminescence [32–34]. Among them, electrochemical analysis of miRNAs has typically attracted growing attention due to their intrinsic advantages, such as high sensitivity, fast response time, simple instrumentation, and low cost [35]. However, the short length, high sequence similarity and low abundance (down to a few molecules per cell) of miRNAs impose difficulty to develop fast, simple, sensitive and selective electrochemical methods for the detection of miRNAs [5,36,37]. Currently, the increasing demand for measuring the ultralow levels of miRNAs with electrochemical techniques is driving the enhancement of sensitivity. Signal amplification by enzyme is one of the most popular strategies for developing ultrasensitive electrochemical genesensors. The method is simple and rapid, but it is still insufficient for detecting the ultra-low concentration of miRNAs by single enzyme amplification [38,39]. Thus, a number of research groups have been exploring various methods to improve the sensitivity by the integrating nanomaterials to further amplify the electrochemical response [40–46]. The methods based on the signal amplification of enzymes-loaded nanomaterials are sensitive, but their practical applications are still limited due to the time-consuming and costly preparation of labeled-nanomaterials. For this view, nanomaterials-free single enzyme amplification would have practical values in the clinical detection of miRNAs.

In the enzyme amplification system, enzymes such as horseradish peroxidase (HRP), glucose oxidase (GOx), and alkaline phosphatase (ALP) are commonly used to enhance the detection sensitivity. Furthermore, gold surface is almost exclusively used for the attachment of thiolated oligodeoxynucleotides (ODNs), forming self-assembled monolayers (SAMs) widely applied in developing DNA/RNA hybridization sensors. We should clarify that HRP and GOx themselves does not directly exchange electrons with the electrode (especially SAMs-covered electrode) because their redox sites are embedded in insulating peptide backbones. So, in this assay, redox mediators have been employed to electronically "wire" the HRP (GOx) reaction center upon contact and catalyze the electroreduction (electrooxidation) of H_2O_2 (glucose) [47–52]. Typically, Wen et al. reported the detection of miRNAs at femtomolar scale using HPR as the label enzyme and 3,3′,5,5′-tetramethylbenzidine (TMB) as the redox mediator [47,48]. Gao demonstrated that miRNAs at the concentration of 25 fM could be readily measured using $Os(bpy)_2(API)Cl$ (bpy = 2,2′-bipyridine, API = 3-aminopropylimidazole)-activated GOx [49]. The $Os(bpy)_2(API)Cl$ moiety bonded to GOx effectively facilitated the transfer of electron from the redox center of $FADH_2$ in GOx to the electrode without altering the biological integrity of GOx. These methods are simple and sensitive, but there still remains significant room to develop more sensitive strategies for miRNAs detection with high simplicity and selectivity since the content of miRNAs in a few biological samples is at the attomolar to femtomolar level.

In the HRP- or GOx-triggered single amplification system, the redox mediator in its oxidation (reduction) form is reduced (oxidized) by the enzyme; then, the oxidized (reduced) enzyme is reduced (oxidized) by the substrate, leading to an increase in the oxidation (reduction) current of the redox mediator. Electrons transferring between redox mediator, enzyme and enzyme substrate (H_2O_2 or glucose) are strictly restricted to the captured enzyme molecules. Thus, such enzyme-linked assays have limitation in term of its sensitivity since one target molecule is usually recognized by one enzyme molecule. Differing from HRP and GOx, ALP is a non-oxidoreductase that hydrolyzes the substrate with no electrochemical activity to a redox substrate. However, because the enzymatic product is not stable in air and the insulating SAMs may cause slow electron transfer between the electrode and the enzymatic product, development of electrochemical miRNAs biosensors with the ALP-based single amplification are limited due to the low detection sensitivity [53]. In the present work, we described an electrochemical–chemical–chemical (ECC) redox cycling for miRNAs detection with ferrocene methanol (FcM) as the redox mediator. In the HRP/GOx-based redox cycling, electrons are transferred between redox mediators and one enzyme molecule (for one target), whereas in the proposed ECC redox cycling electrons are transferred between redox mediators and thousands of enzymatic products (even for one target). Furthermore, the enzymatic products can be regenerated immediately by the extra chemical reducing reagent after their oxidation. The result indicated that this method was very sensitive. To demonstrate the feasibility of our method for analysis of miRNAs in real samples, the expression levels of miRNA-21 in human serum samples were tested.

2. Experimental

2.1. Chemical and reagents

Streptavidin-conjugated alkaline phosphatase (SA-ALP), 6-mercaptohexanol (MCH), tris(carboxyethyl)phosphine (TCEP), bovine serum albumin (BSA), L-ascorbic acid 2-phosphate (AAP), ferrocene methanol (FcM) and tris-(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) were obtained from Sigma–Aldrich (Shanghai, China). Diethylpyrocarbonate, dimethyl sulfoxide (DMSO) and hairpin-like DNA probe modified with a thiol and a biotin (5′-SH-$(CH_2)_6$-CCGAAGTCGACTCAACATCAGTCTGATAAGC-TAGAAGTCGACTTC-biotin-3′) were purchased from Sangon Biotech. Co., Ltd. (Shanghai, China). The miRNAs were obtained from GenePharma Co., Ltd. (Shanghai, China). Their sequences are 5′-UAGCUUAUCAGACUGAUGUUGA-3′ (miRNA-21), 5′-UAGCUUAUCGGACUGAUGUUGA-3′ (single-base mismatch), 5′-UUGCUUAUCGGACUGAUCUUGA-3′ (three-base mismatch) and 5′-GUAAGGCAUCUGACCGAAGGCA-3′ (non-complementary). Human serum samples from one staff and two students were obtained from the health center of Anyang Normal University (Anyang, China). Other chemicals were analytical-grade reagents and were used without further purification.

The DNA solutions were prepared using TE buffer solution (10 mM Tris–HCl, 1 mM EDTA, pH 7.4), and kept at −18 °C. The miRNAs stock solutions were prepared daily with TE buffer solution in an RNase-free environment. The stock solution of FcM (10 mM) was prepared with DMSO and then diluted with Tris buffer solution (10 mM, pH 8.0) to the desired concentration. All of

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the solution and redistilled deionized water used were treated with diethylpyrocarbonate and autoclaved to protect from RNase degradation.

2.2. Instruments

Electrochemical determination was carried out on a CHI 660E electrochemical workstation (CH Instruments, Shanghai, China) with a platinum wire and a Ag/AgCl electrode as the auxiliary and the reference electrodes, respectively. Quantitative real time polymerase chain reaction (qRT-PCR) experiments were performed on a LightCycler Nano Real Time PCR Instrument (Roche, UK).

2.3. Preparation of DNA/MCH-covered electrode

The gold disk electrode with 2-mm-diameter was polished sequentially with 0.3 μm and 0.05 μm alumina, followed by ultrasonic cleaning in ethanol and water. Then, the electrode was treated electrochemically by cycling the potential in the range of −0.3 to +1.5 V in 0.5 M H_2SO_4 solution. The cleaned gold electrode was then immersed in the TE buffer solutions containing 1 μM hairpin DNA probe, 0.1 M NaCl and 50 μM TCEP and kept overnight at 4 °C. This step was followed by washing the electrode thoroughly with water and soaking it in a 0.1 mM MCH solution for 20 min to block the unreacted gold surface and hold a good orientation of the DNA probe for its good recognition ability.

2.4. Procedure for miRNA-21 detection

For the assays of miRNA-21, the DNA/MCH sensing electrode was incubated with 50 μL of TE buffer solution containing 0.1 M NaCl, 1 μM SA-ALP and a given concentration of miRNA-21 at 37 °C for the DNA/miRAN-21 hybridization and the attachment of SA-ALP. In order to minimize adsorption of the enzyme on the electrode and thus to improve the reproducibility of the results during the homogeneous electrochemical studies, BSA was included in the TE buffer solutions at a concentration of 1 mg mL^{-1}. It was then rinsed thoroughly with the hybridization buffer and water after 60 min. Thereafter, the resulting sensing electrode was incubated with 20 μL of Tris buffer solution (10 mM, pH 8.0) in the presence of 0.5 mM AAP and 1 mM $MgCl_2$ in the homemade plastic electrochemical cell. After 30 min, 20 μL of Tris buffer solution containing 0.5 mM TCEP and 0.2 mM FcM was added into the electrochemical cell. Finally, voltammetric or amperometric detection was carried out on the CHI 660E electrochemical workstation electrochemical workstation. After each assay, the electrode was regenerated by incubation of the sensing electrode in hot water (90 °C) for 10 min to desorb the target miRNAs. Then, the electrode was rinsed with 5% DMSO/water, incubated with the TE buffer solution containing 0.1 M NaCl, and washed with deionized water.

For the assays of miRNA-21 in serum samples, 0.1 mL of serum was mixed with 0.1 mL TE buffer solution. Then, NaCl, BSA and SA-ALP were added to the sample solution to bring the final concentrations to 0.1 M, 1 mg mL^{-1} and 1 μM, respectively.

3. Results and discussion

3.1. Principle of this method

Molecular beacon composing of a hairpin-like DNA stem-loop structure has been widely used for nucleic acid detection with excellent sensitivity and selectivity [54–56]. The hybridization event induces the conformational change of the gold surface-immobilized hairpin DNA probe. In this paper, we developed a hairpin DNA probe-based "signal-on" biosensor for miRNAs detection. As shown in Fig. 1, the hairpin DNA probe modified with a thiol group at the 5′-end and a biotin group at the 3′-end was immobilized onto the gold electrode surface. In the absence of target miRNAs, steric hindrance prevented binding of SA-ALP to the biotin-labeled end of the hairpin probe located close to the electrode surface. Upon contact with the target miRNAs, the hairpin structure was destroyed and the biotin group was forced away from the surface of electrode, which allowed for the coupling of SA-ALP. Then, the enzymatic products triggered the ECC redox cycling. In the detection system, FcM, TCEP and AAP were used as the redox mediator, reducing reagent and enzyme substrate respectively because: (1) ferrocene derivatives that have been used as the mediators to electronically "wire" the enzyme reaction center are relatively stable in air and are able to be regenerated after its electrochemical-oxidation by the ALP enzymatic products during the electrochemical scanning [57,58], (2) TCEP, a stable and electrochemically inert reducing regent showing no reaction with the oxidized form of ferrocene, can regenerate AA from dehydroascorbic acid (DAA, the oxidation form of AA) [59,60], and (3) among kinds of ALP enzyme substrates, AAP is better because of its low cost, the easy dissolution of AAP and AA in

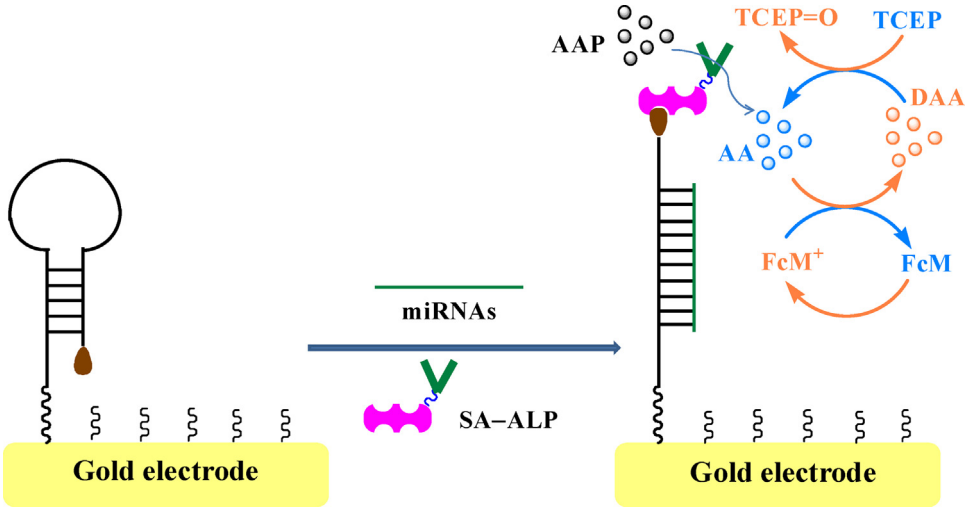

Fig. 1. Schematic illustration of the strategy for miRNAs detection using the hairpin DNA probe based on the signal amplification of ALP and ECC redox cycling.

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aqueous solutions, the high formal potential of AAP and low formal potential of AA and the high signal-to-background ratio [60]. Moreover, we found that the insulating SAMs can hinder the electro-oxidation of AA although AA is a strong reducing reagent. In these terms, SA-ALP captured by the electrode would catalyze the production of large amounts of AA, triggering the ECC redox cycling. Specifically, FcM was first electrochemically oxidized to ferricinum ion (FcM^+) and then regenerated by the enzymatically produced AA. In this process, AA was also regenerated immediately from its oxidation production DAA by TCEP, thus enhancing the anodic current of FcM. Because of the accumulation of thousands of AA molecules, one target miRNA molecule would initiate a plurality of redox reactions in the ECC recycling. Thus, we believe that the method based on the single enzyme amplification plus the ECC redox cycling would be very sensitive and particularly popular in bioassays because it only requires the addition of more chemicals to the substrate solution and not a change in the detection procedure of conventional enzyme-based assays. To demonstrate the feasibility and sensitivity of this method, miRNA-21 was determined as a model analyte.

3.2. Feasibility

Fig. 2 shows the CVs of the DNA/MCH-covered electrode after the hybridization of miRNA-21, the capture of SA-ALP, and the follow-up incubation with different substrate solutions. It can be observed that no redox peaks were observed in the solutions of AAP (curve a) and AAP/TCEP (curve b), indicating that the insulating DNA/MCH SAMs hindered the electro-oxidation of AA. In the FcM system, the generated AA caused a significant increase in the anodic current of FcM, which was accompanied by the decrease in its cathodic current (cf. curves c and e). The electrode reaction is characteristic of an electrochemical–chemical (EC) reaction mechanism, indicating that FcM mediated the electron transfer between AA and electrode. Furthermore, the presence of TCEP induced a more significant increase in the anodic current (curve f). The result can be attributed to the regeneration of AA from its oxidized product by TCEP since TCEP itself did not induce a change in the anodic current of FcM (cf. curves c and d). These results demonstrated that the EC reaction between FcM and AA was promoted by the chemical–chemical (CC) reaction between AA and TCEP, thus, amplifying the electrochemical signal. Additionally, we found that no potential change was observed for FcM with the variety of scanning rate whereas the potential difference ($\Delta E = E_{pa} - E_{pc}$) increased with the increase of scanning rate in the case of $[Fe(CN)_6]^{3-}$ (see Fig. S1 in Supplementary material). Moreover, the electrode reaction of $[Fe(CN)_6]^{3-}$ on the modified electrode was irreversible. The results were understandable since the neutral insulating SAMs were excluded from the negatively charged $[Fe(CN)_6]^{3-}$ probe and formed a barrier for the electron transfer. Nevertheless, the well-defined FcM voltammetric peaks implied that the SAMs have no influence on the electron transfer of FcM probably due to the hydrophobic interaction between FcM and SAMs. These results also demonstrated that the electrode reaction of FcM at the electrode surface is a typical adsorption-controlled process. Furthermore, although ferrocenecarboxylic acid (FcA) is a water-soluble ferrocene derivative that has been used as the redox mediator of many enzymes, we also found that the method with FcA as the mediator is less sensitive than that with FcM in the AA-triggered recycling system (Fig. S2). Thus, FcM was used as the redox mediator in this work.

3.3. Regeneration and reproducibility

Because of the instability and low abundance of miRNAs in biological matrixes, considerable efforts have been devoted to develope high-throughput analytical strategies for multiplexed miRNAs gene expression, including Northern blotting and microarray-based detection platforms. It has been reported that the hairpin DNA-based electrochemical genesensors could be regenerated by incubating the sensing electrode in hot water (90 °C) [56,61]. In this process, the hybridized miRNAs were removed *via* thermal denaturation. After washing the electrode with hybridization buffer, the DNA probe forms a hairpin structure again, which allows for the reuse of the sensing electrode. Herein, we also found that the electrode can be conveniently regenerated by incubating the electrode in hot water for 10 min. As shown in Fig. 3, no obvious change in both the response and background currents was observed after ten regeneration/assay cycles. Thus, multiple samples can be determined using one electrode, dramatically increasing the sample throughput and reducing the sample analysis time. Moreover, the accumulation, polymer formation and poor dissolution of the ALP enzyme substrates/products (*e.g.*, quinone derivatives) on the electrode surface may lead to the passivation of electrode in electrochemical assays. The good regeneration in this work also indicated that AAP/AA did not

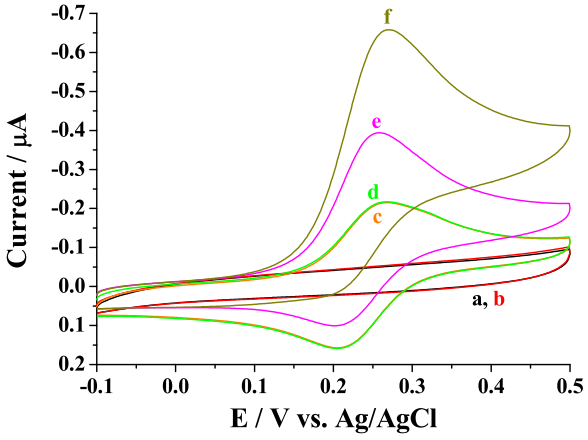

Fig. 2. CVs of the DNA/MCH-modified electrode after incubation with the miRNA-21/SA-ALP solution and follow-up incubation with different substrates: curve a, AAP; curve b, AAP/TCEP; curve c, FcM; curve d, TCEP/FcM; curve e, AAP/FcM; and curve f, AAP/TCEP/FcM. The concentration of miRNA-21 was 50 pM. The scan rate was 20 mV s^{-1}.

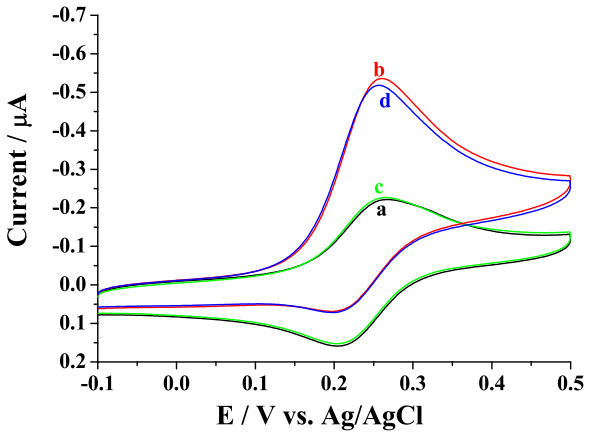

Fig. 3. CVs of the DNA/MCH-modified electrode after incubation with miRNA-21/SA-ALP and follow-up incubation with FcM (curves a and c) and AAP/TCEP/FcM (curves b and d) before (curves a and b) and after (curves c and d) regeneration. The concentration of miRNA-21 was 20 pM. The other experimental conditions are the same as those in Fig. 2.

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passivate the sensing electrode. This is attributed to the easy dissolution of AAP and AA in aqueous solutions. Reproducibility is an extremely important feature of biosensors in their practical applications. To investigate the reproducibility of this method, three electrodes with the same background current were fabricated independently in the same conditions and used for the assay of miRNA-21. The relative standard deviation (RSD) was found to be ~8.5%, indicating that multiple electrodes can be prepared concurrently for the assays of many different samples.

3.4. Sensitivity

The quantitative behavior was also assessed by monitoring the dependence of the amperometric current upon the amount of miRNA-21. Experiments were carried out by adding increasing amounts of miRNA-21 to the SA-ALP solution to examine whether the current change could be used for miRNA-21 quantification. Fig. 4 shows the amperometric current curves of the sensor upon the presence of different amounts of miRNA-21. It can be seen that the currents increased significantly with the increasing miRNA-21 concentrations. For quantification, we measured the amperometric current variation Δi ($\Delta i = i - i_0$, where i_0 and i represent the amperometric current in the absence and presence of miRNA-21). A good linear relationship between Δi and miRNA-21 concentration was from 0.5 fM to 1 pM with a linear regression equation of Δi (μA) = 0.001 + 0.019 [miRNA-21] (pM) (R^2 = 0.99). The detection limit estimated from 3s of the baseline signals was 0.2 fM. This value is lower than that achieved by other enzyme-amplified electrochemical strategies, such as Os $(bpy)_2$(API)Cl-activated GO (4 fM) [49], HPR with TMB as a redox mediator (10 fM) [47], HRP/AuNPs/graphene (6 fM) [40], ALP/paramagnetic beads (7 pM) [44], AuNPs/ALP/redox cycling (3 fM) [42], and Pd–Pt supported graphene/ALP/redox cycling (3.55 fM) [45]. The lower detection limit is attributed to the dual signal amplification of ALP and the ECC redox cycling. Moreover, our strategy is technically simple and obviated the use of nanomaterials or multi-enzymes for signal amplification, reducing the operation complexity and assay cost.

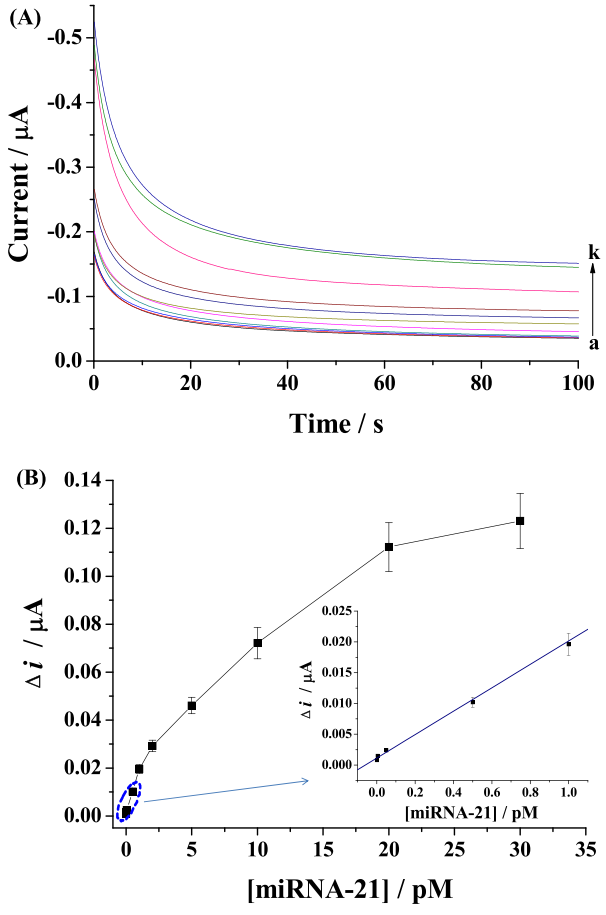

Fig. 4. (A) Amperometric response of the DNA/MCH-modified electrode in the solution of AAP/TCEP/FcM after hybridization of miRNA-21 and attachment of SA-ALP. The concentrations of miRNA-21 varied in the range of 0–30 pM (from a to k: 0 fM, 0.5 fM, 5 fM, 50 fM, 0.5 pM, 1 pM, 2 pM, 5 pM, 10 pM, 20 pM and 30 pM). The amperometric *i*–*t* curves were measured at 0.3 V. The quiet time was 2 s. (B) Dependence of Δi on the miRNA-21 concentration. The current values were collected at 100 s. The inset shows the linear segment in the range of 0.5 fM–1 pM. Each point was averaged from at least three replicates, and the error bars show the absolute standards.

3.5. Specificity

The specificity of the hairpin DNA-based assay method was investigated by using the single-base mismatched and three-base mismatched miRNAs as well as a complete non-complementary strand. As shown in Fig. 5, the current change in the cases of three-base mismatched and non-complementary strands was negligible, indicating that the hairpin structure was not opened by these two strands. In the case of single-base mismatched strand, we found that Δi were ~15% of that from the miRNA-21 target, suggesting that the novel assay has excellent specificity to distinguish the single-base mismatched target, which benefits from the use of the stem-loop DNA probe as the sensing component in this approach. The high specificity is very significative for miRNAs assays in clinical application in view of the short length and high sequence similarity of miRNAs in a biological matrix.

3.6. Human serum samples

To demonstrate the amenability of our method to miRNAs analysis in real samples, the expression levels of miRNA-21 in human serum samples extracted from 3 donors were tested. As shown in Fig. 6, the contents of miRNA-21 were found to be 3.8 fM, 5.7 fM and 6.2 fM by the calibration curve, respectively. To ascertain the correctness of the results, we also tested the

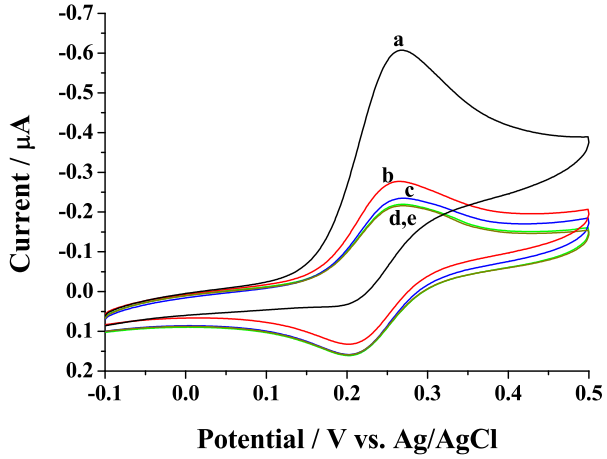

Fig. 5. CVs of the DNA/MCH-modified electrode after incubation with the mixture of SA-ALP and different miRNAs strands (curve a: miRNA-21; b: single-base mismatch; c: three-base mismatch; d: non-complementary; and e: blank sample) and follow-up incubation with AAP/TCEP/FcM. The concentration of all miRNAs strands was 30 pM.

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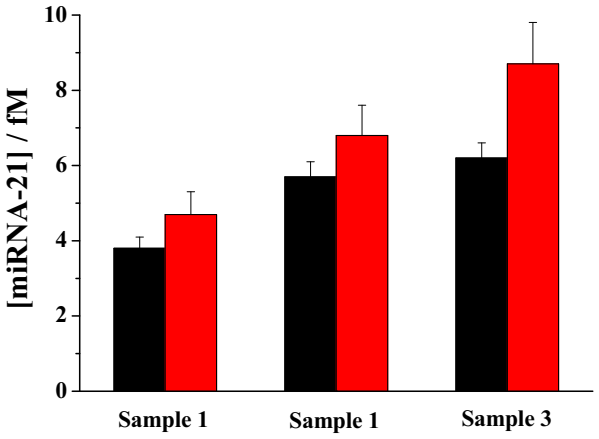

Fig. 6. Expression levels of miRNA-21 in three serum samples detected by the presented method (black bars) and qRT-PCR (red bars), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

miRNA-21 contents with qRT-PCR. The results shown in Fig. 6 indicated that the levels of miRNAs in three real samples obtained by our method and qRT-PCR were of the same order of magnitude. Thus, the electrochemical biosensor described herein is applicable for miRNA-21 detection in real samples and can offer a useful means for quantifying miRNAs in clinical studies. These results also demonstrated that all the three donors are healthy since the basal level of miRNA-21 in normal human serum is at femtomolar scale [3]. But even so, the values from the electrochemical results were slightly lower than those obtained by qRT-PCR statistically. A reasonable excuse for this point was that the biological matrixes decreased the analytical performances of the sensing electrode.

4. Conclusion

In summary, we developed a simple, sensitive and selective electrochemical biosensor for miRNAs detection based on single enzyme amplification and ECC redox cycling. The ECC redox cycling was triggered by the enzymatic product AA using FcA and TCEP as the redox mediator and the reducing reagent, respectively. Due to the accumulation of large amounts of AA during the enzymatic process, the detection limit of this method (0.2 fM) was lower than that obtained by the single enzyme (HRP or GOx) amplification. Moreover, this biosensor only requires the addition of more chemicals to the substrate solution and obviates the use of nanomaterials for loading multi-enzymes, reducing the operation complexity and assay cost. The method described herein was also extended to the quantification of miRNA-21 present in human serum samples from three donors. The results are consistent with those obtained by qRT-PCR. The simple operation procedure, high sensitivity and specificity as well as good regeneration and reproducibility of this method indicated that our work would be valuable for quantifying miRNAs in clinical diagnostic and prognostic and designing new types of electrochemical biosensors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2015.04.018.

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